Regulation of the proliferation and osteogenic differentiation of colony forming units-fibroblastic derived from human bone marrow

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Regulation of the Proliferation and Osteogenic Differentiation of Colony Forming Units-Fibroblastic Derived from Human Bone Marrow

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SUMMARY AND AIMS

The World Health Organisation defines osteoporosis as a bone mineral density which is more than 2.5 standard deviations below the young adult mean. This deficit of bone tissue is widespread in older members of the population and is a cause of increased fragility and fracture risk. Injuries resulting from this insufficiency are a major cause of pain, deformity, lost independence and morbidity and represents a considerable drain on Health Service resources.

It is accepted that osteoporosis is caused by a focal imbalance in the bone remodelling cycle and, in the early stages of the post menopausal variant, the pattern of loss is consistent with an increase in bone resorption. More recent evidence however suggests that bone loss in the later stages of the disease and in all other forms of osteoporosis, is due to a reduction in bone formation at the tissue level. This reduction appears to be due to a decline in the number of osteoblasts, the cause of which is currently unknown.

In the post-natal organism, osteoblasts are derived from clonogenic precursors (CFU-F) associated with the soft fibrous tissue of the marrow stroma. Studies have shown that a sub-set of the CFU-F population is capable of giving rise to all of the cell lineages necessary for bone formation and the recreation of the haematopoietic micro environment.

One mechanism that has been postulated to account for the decline in osteoblast number is that an age related reduction in the number of CFU-F causes a reduction in the number of potential osteogenic precursors. Identifying the causes of the osteoblast deficit in osteoporosis ultimately depends on an improved understanding of the factors that induce the promotion of uncommitted cells to committed osteoprogenitors and regulate the regulate the size of the CFU-F population.

As these hypotheses are largely based on studies in animals or animal bone cells, the purpose of this project is to investigate these possibilities using cells derived from adult human bone marrow. The specific aims are to identify factors that promote the proliferation and osteogenic differentiation of human bone marrow CFU-F

ACKNOWLEDGEMENTS.

I am glad of this opportunity to warmly thank my supervisor Dr Jon Beresford, firstly, for giving me the chance to undertake this project, but particularly for his help and support throughout my time at B.I.R.D.. Jon's "open door" policy coupled with his extensive knowledge of bone cell biology, meant that valuable advice and encouragement were always at hand.

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ABBREVIATIONS.

AA	Amino acid.
AC	Adenylate cyclase.
AMP	Adenosine-5'-monophosphate
AP	Alkaline phosphatase.
Asp	Ascorbic-2-phosphate.
ATP	Adenosine triphosphate.
BMP	Bone morphogenetic protein.
C	Centigrade.
Ca^{++}	Calcium
cAMP	Cyclic adenosine-5'-monophosphate.
cDNA	Complementary deoxyribonucleic acid.
CFU-F	Colony forming unit- fibroblastic.
CFU-GM	Colony forming unit- granulocyte macrophage.
CFU-M	Colony forming unit- macrophage.
cm	Centimetre.
CO_2	Carbon dioxide.
CSF	Colony stimulating factor
D_3	1,-25-dihydroxy-vitamin D_3.
DAG	Diacylglycerol.
DMEM	Dulbecco's modified Eagles medium.
Dx	Dexamethasone.
DNA	Deoxyribonucleic acid.
EGF	Epidermal growth factor.
ER	Endoplasmic reticulum
FACS	Fluoroscene activated cell sorting.
FCS	Fetal calf serum.
FGF	Fibroblast growth factor.
FGFr	Fibroblast growth factor receptor.
GDP	Guanine diphosphate
GTP	Guanine triphosphate
GLA	Gamma carboxyglutamic acid.
h	Human.
HCl	Hydrochloric acid.
HRE	Hormone receptor element.
HRC	Hormone receptor complex.
IBMX	Methyl-iso-butyl-xanthine.
IGF	Insulin-like growth factor.
IL-	Interleukin
IP3	Inositol 1,4,5 trisphosphate.
M	Molar.
MAD	Mothers against decapentaplegic.
MGP	Matrix gla protein.
ml	Millilitre.
mm	Millimetre.
MNC	Mononuclear cells.
MTT	3-(4,5 Dimethylthiazol-2y)-2,5-diphenyl tetrazolium bromide
NCP	Non collagenous proteins.

mM	Millimolar.
nM	Nanomolar.
nm	Nanometre.
μM	Micromolar.
OAF	Osteoclast activating factor
OC	Osteocalcin.
OPN	Osteopontin.
P	Phosphate
PBS	Phosphate buffered saline.
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor.
PI	Phosphoinositol
PIP2	Phosphatidylinositol 4, 5 bisphosphate.
PKA	Protein kinase A.
PKC	Protein kinase C.
PLC	Phospholipase C.
PTH	Parathyroid hormone.
PDGF	Platelet-derived growth factor.
PPP	Platelet poor plasma.
RER	Rough endoplasmic reticulum
RTPCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate.
SFM	Serum free media.
SLR	Single lens reflex.
Smad	Human homologue of MAD.
TGFα	Transforming growth factor alpha.
TGFβ	Transforming growth factor beta.
TGFr	Transforming growth factor receptor.
TNF	Tumour necrosis factor.
TNSAP	Tissue non-specific alkaline phosphatase

Chapter 1: Introduction

1.1: Osteoporosis.

The importance of improving the current understanding and management of osteoporosis becomes obvious when the extent and effects of this disorder are considered.

Osteoporosis may be defined as one of a widespread heterogeneous group of disorders characterised by decreased bone mass and abnormal bone remodelling[1]. The condition causes an increase in the risk of fractures to the femoral neck, vertebrae and radius causing loss of independence, deformity and pain. Complications arising from these injuries are also the cause of great morbidity, 15% of older women sustaining femoral neck fractures, die within 6 months from resultant complications[2].

Economically, the 60, 000 hip fractures, 50, 000 wrist and 40, 000 vertebal fractures currently attributable to osteoporosis are costing the health service an estimated £750 million per annum[3].

Similar to many pathological conditions, osteoporosis is a multifactorial disease and is categorised either as Primary (with sub-types 1 and 2) or Secondary. Table 1.1 below, summarises the important differences between them.

Table 1.1

Primary Osteoporosis

Type	Affects	Cause	Bone Loss	Fractures
1	Post-menopausal women	Oestrogen deficiency	Mainly Trabecular	Vertebral + Radial
2	Men + Women	Ageing	Cortical +Trabecular	Hip

Secondary Osteoporosis, Some Causes.

Alcohol, Smoking, Cushing's Syndrome, Hypogonadism, Menstrual dysfunction, Hyperthyroidism, Anorexia nervosa, Excessive exercise, Pregnancy, Rheumatoid Arthritis, Myeloma, Chronic liver disease, Malabsorption, Systemic Mastocytosis

Common to all types of osteoporosis is a focal imbalance between the activity of osteoclasts and osteoblasts during the remodelling cycle that results in a net loss of bone. In normal adults 25% of cancellous bone is resorbed and replaced every year, compared with only 3% of cortical bone[4]. Therefore in osteoporosis, it can be expected that cancellous bone will endure erosion to a greater extent.

1.1.1: Bone Metabolism: Influencing Factors.

The focal imbalance in osteoporosis can be caused by:

Increased resorption, formation normal

Normal resorption, formation reduced

Increased resorption and formation but the former more than the latter

Decreased resorption and formation but the latter more than the former

Increased resorption may be caused by:

(i) Increased rates of osteoclastic activity
(at the expense of osteoblastic activity)

(ii) Increased frequency of activation of absorption sites

Reduced formation may be caused by:

(i) Altered function of osteoblasts

(ii) Increased depth of resorption cavities with loss of template

(iii) Impaired recruitment of osteoblasts.

As osteoporosis is associated with local changes in trabecular bone mass and structure, any of the above situations could result from a disturbance of the local microenvironment or in the levels of systemic hormones known influence the activation frequency[5-7].

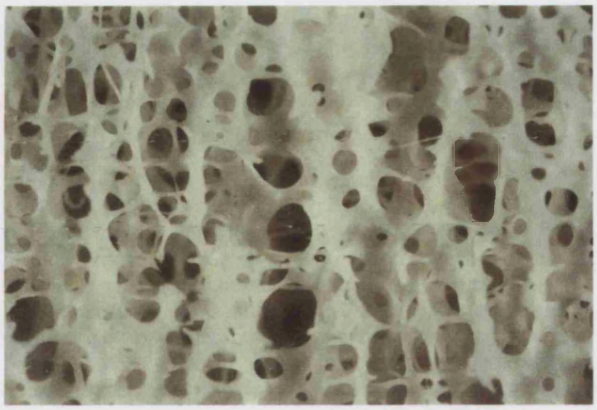
Figure 1.1:

(a) Normal vertebrae

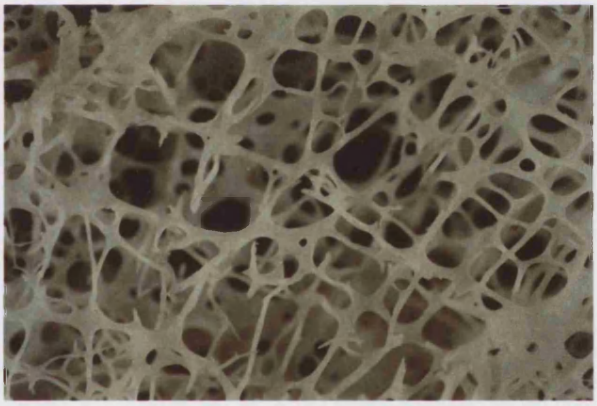
(b) Osteoporotic vertebrae

(c) Severely osteoporotic vertebrae
Note the loss of trabecular continuity
due to resorptive perforations.

Figure 1.1: Scanning Electron Micrographs of Trabecular Bone Obtained from (a) Normal (b) Osteoporotic and (c) Severely Osteoporotic Vertebrae.



(a)



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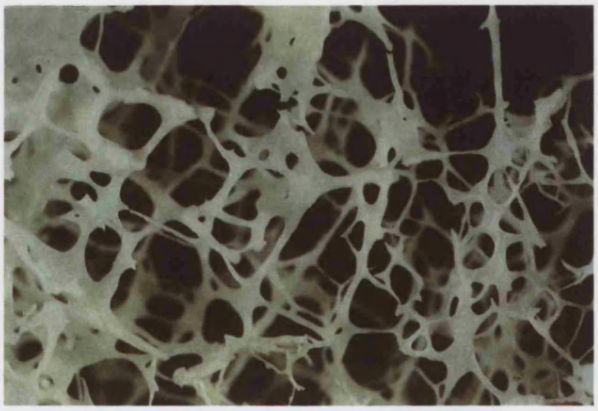


(c)

Taken from; Eriksen, E.F. Bone Histomorphometry.
New York, Raven Press: 1994

1.2: Bone.

Bony, or osseous tissue, is a specialised connective tissue that combines the strength of cast iron with the lightness of wood. Functionally, bone serves three main purposes.

(i) Mechanical; providing support and muscle attachment sites required for locomotion.

(ii) Protective; safeguarding vital organs such as brain, spinal cord and bone marrow from external trauma.

(iii) Storage; serving as a site for reserves of calcium and phosphate.

Additionally, the bone marrow is the site of haematopoesis in the post-natal organism and source of connective tissue stem cells. It is important to emphasize that bone is not a dry, lifeless, brittle structure, but that, on the contrary, it is a living, dynamic, productive tissue that is continually formed, resorbed and reformed .

Like other sub-types of connective tissue, bone is composed of cells embedded in a matrix of osteoid and fibres[8]. Bone rigidity is provided by the deposition of inorganic salts such as calcium phosphate and calcium carbonate in the osteoid. Once deposited these substances undergo phase transitions and develop into a form of hydroxyapatite which may be ionised when required to provide calcium and phosphate to satisfy transient bodily needs. The organisation of collagenous fibres in the matrix contributes towards bone strength and flexibility.

1.2.1: Bone Cells.

Four types of bone cell comprise the cellular component of bone and they have the ability to modify their function in response to bodily demands.

Osteoblasts are metabolically active cells found on the developing surfaces of bone and are the principal cells of bone formation. Osteoblasts arise from osteoprogenitor cells of mesenchymal origin (pre-osteoblasts) and are highly differentiated and non-dividing cells[9].

Histologically, the mature osteoblast is cuboidal in shape and possesses a polarised arrangement of organelles[10] (fig. 1.2). Extensive amounts of rough endoplasmic reticulum (RER) are found clustered in the cytoplasm nearest the bone surface and at the opposing end of the cell lies the nucleus containing 1-3 nucleoli. Sandwiched between the nucleus and the RER is found a prominent Golgi apparatus. The deep basophilic staining associated with osteoblasts is related to the abundance of glycogen vesicles which fuel the metabolic needs of the cell. Osteoblasts also stain positively for the enzyme alkaline phosphatase (discussed below) which is localised to the plasma membrane.

The developmental progress from stem cell to the osteoblast phenotype is marked by several stages of differentiation. Current knowledge of the nature of these commitment steps is however, still relatively poor. Using morphological and histochemical criteria coupled to analysis of proliferation, four maturational stages have been identified in bone *in situ* - pre-osteoblast, osteoblast, osteocyte and bone lining cell[11]. By these criteria, osteoblasts are recognizable as post-proliferative and cuboidal, strongly alkaline phosphatase positive cells in contact with bone matrix at sites of osteogenesis.

The pre-osteoblast is recognised as the immediate precursor of the osteoblast and is identified by its position, one to two cell layers behind the osteoblast front. Although pre-osteoblasts resemble osteoblasts histologically and ultrastructurally and also express for alkaline phosphatase, they have not acquired many of the other differentiated features of fully mature cells[12]. Figure 1.3 shows a widely accepted view of the phenotypic stages associated with osteoblast differentiation. The principle function of the osteoblast is to synthesise bone matrix facilitating the production of new bone. Synthesis of new bone occurs in 4 sequential steps:

(i) Synthesis and intracellular processing of type I collagen and non-collagenous proteins.
(ii) Secretion and extracellular processing of the collagen and non-collagenous proteins.
(iii) Formation of microfibrils, fibrils and fibres from the collagen.
(iv) Maturation of the collagen matrix (osteoid) with subsequent nucleation and growth of hydroxyapatite crystals (mineralisation)[10].

Figure 1.2: Schematic Diagram of Osteoblast Ultrastructure.

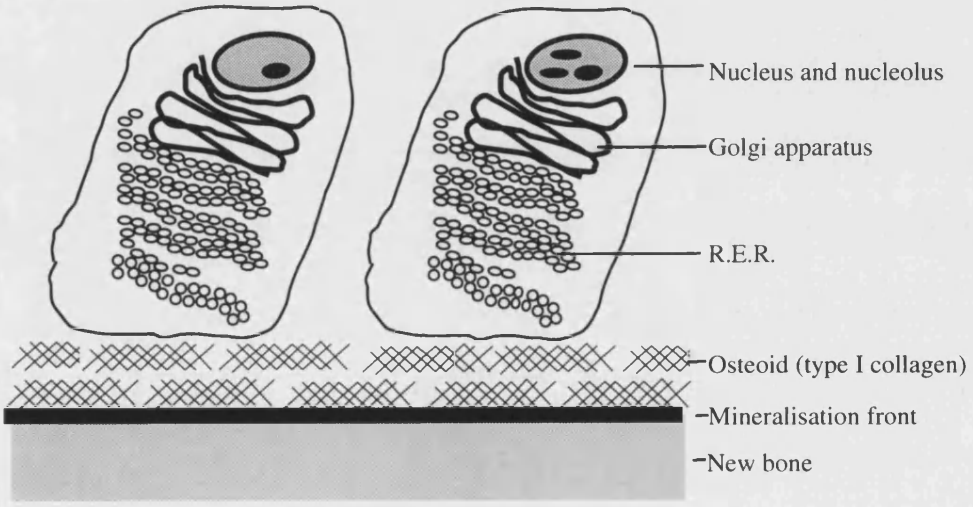
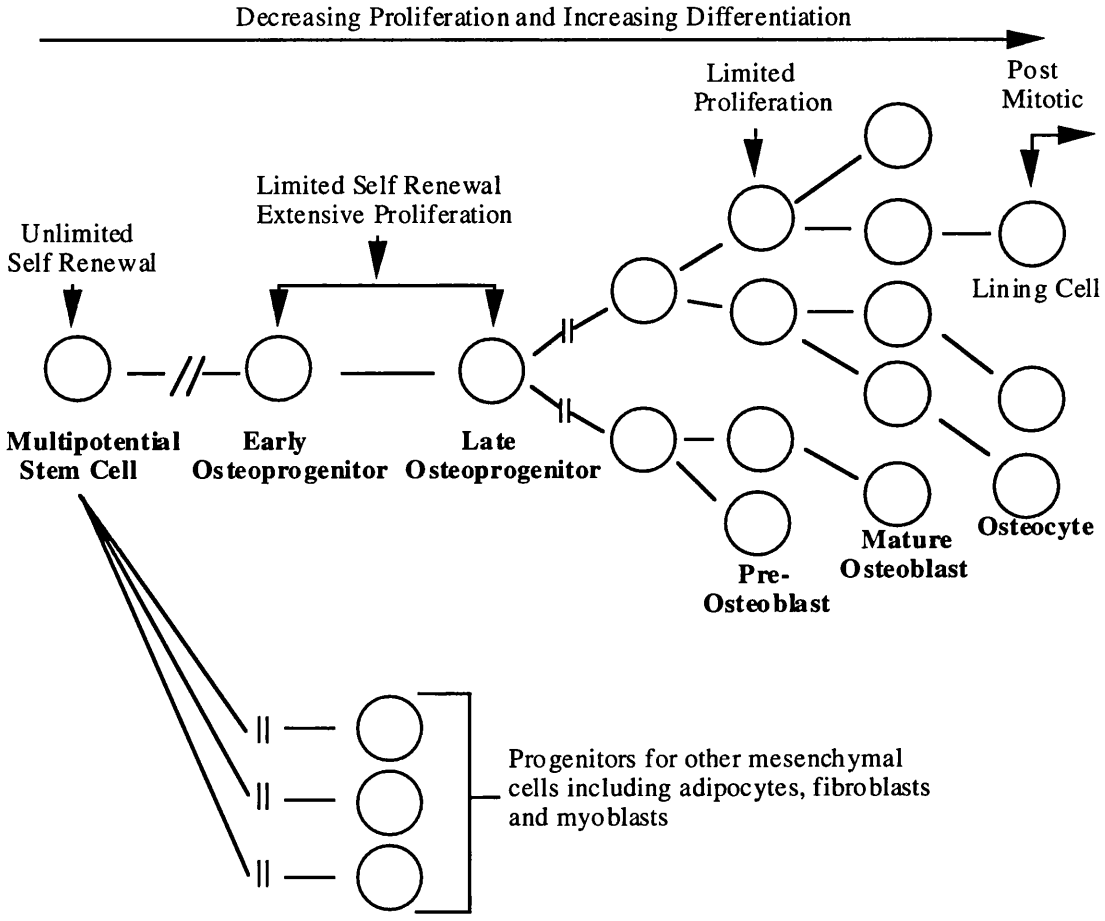


Figure 1.3: Steps in Osteoblast Lineage Implying Recognizable Stages of Differentiation (Adapted from Aubin [12]).



The mechanism of mineralisation is not yet fully understood but it is accepted that it is an extracellular event. In woven bone, cartilage, dentin and cementum the onset of mineralisation is frequently associated with the appearance of vesicular structures termed matrix vesicles. These are typically located some distance from the cells where mineralisation is to occur. They measure about 100nm in diameter and are formed by the pinching off of plasma membrane from the cells producing the mineralised tissue (e.g.. osteoblasts). Matrix vesicles contain alkaline phosphatase and other enzymes that are considered to load the membrane of the vesicle, then the vesicle itself with calcium and phosphate. It is thought that on rupturing, the vesicle causes a local increase in mineral concentrations sufficient to initiate mineralisation[9]. In lamellar bone the mechanism of mineralisation appears to be different. Mineralisation is initiated in the gap between overlapping collagen molecules (fig. 1. 7) where there are few matrix vesicles and appears to be initiated by components of the collagen itself or non-collagenous proteins at this site[13]. It is known that the mineral in mature calcified bone consists of carbonated hydroxyapatite crystals approximately 1.5 nm X 30 nm and it is thought that these needle shaped structures align with the collagen fibrils.

Throughout the osteogenic process, osteoblasts are influenced by both systemic hormones and, local agents in the form of growth factors, cytokines and other short lived diffusable mediators e.g. prostaglandins and nitric oxide. Interestingly, it has been postulated that the paracrine regulation of bone formation is dependant on, at least in part, the release and activation of matrix bound factors deposited during previous rounds of bone formation.

As bone formation proceeds, a subset of osteoblasts differentiate further into a relatively inactive flattened cell known as a bone lining cell, while others become entombed in a mineralised matrix of their own making. When the osteoblast becomes totally encased, access to nutrients becomes severely restricted resulting in a concomitant reduction in metabolic activity. At this stage the enclosed osteoblast becomes known as an osteocyte.

Bone Lining Cells are osteoblasts which have undergone an extra stage of differentiation and are found on the endosteal surface of most bones. A major function of these cells is thought to involve the establishment of an ion barrier around bone tissue, thus regulating the influx and outflow of calcium and phosphate to and

from the matrix[14]. Recent work has suggested that these cells may also have the ability to dedifferentiate,[15] providing a ready source of osteoblasts at times of crises, e.g.. during fracture repair[16]. Bone lining cells may also exert an influence on bone remodelling by regulating osteoclast access to bone surfaces.

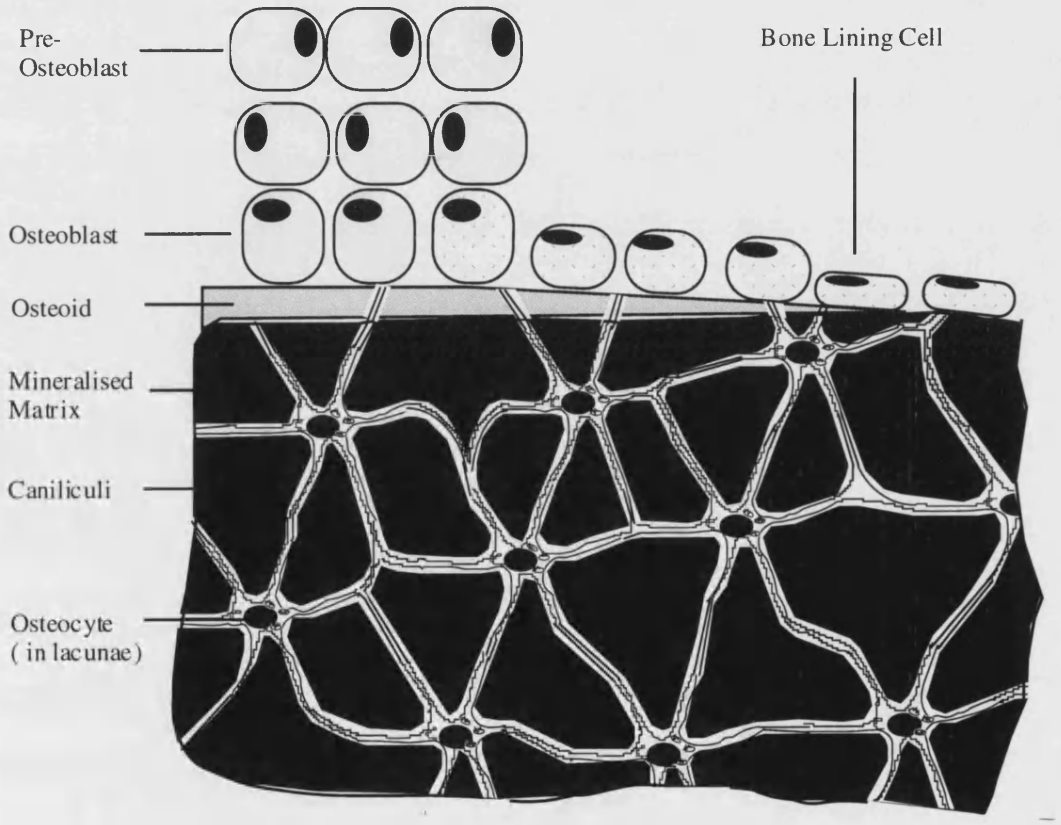
Osteocytes are found in lacunae, small cavities in the calcified matrix and, are the most abundant cell type in bone. It is estimated that osteocytes exceed osteoblast numbers by approximately 10 fold [17].

Histologically, osteocytes appear as cells with a large nucleus to cytoplasmic ratio, resulting in a thin rim of cytoplasm. Long cytoplasmic extensions give the cell a stellate appearance allowing nutritional access and intercommunication between adjacent osteocytes within the matrix. Osteoblasts and bone lining cells on the bone surface are also accessible via this network (fig. 1. 4). The cytoplasmic extensions are routed through the surrounding matrix via minute channels known as *canaliculi* [9]. Transmission electron microscopical (TEM) examination of the cell reveals a paucity of RER and diminished Golgi complex, reflecting a reduced level of cellular function [18].

Osteocytes are derived from the same multipotential mesenchymal stem cells as the osteoblast and represent an alternative state of terminal differentiation of the osteoblast lineage. The mechanism by which osteoblasts differentiate into osteocytes is not well understood. During bone formation, a proportion of osteoblasts lining the surface of bone are left behind and become incorporated into the newly laid osteoid matrix while other osteoblasts move on. It is thought that 10-20% of osteoblasts become embedded in a mineralised matrix of their own making[19].

It is thought the osteocyte performs several roles. One is the maintenance of bone matrix, utilising a limited capacity to synthesise and resorb matrix. Death of an osteocyte results in the resorption of the matrix by osteoclastic activity followed by the repair or remodelling of the tissue by osteoblasts[9]. Evidence suggests that osteocytes are also the mechanosensory cells of bone and play a central role in its dynamic adaptation to changes in mechanical loading[20]. Mechanical stress on

Figure 1.4: Schematic Representation of Relationship between Bone Cells and Matrix.



bone results in a very small deformation, or strain on bone tissue. This deformation of the bone matrix then results in a flow of interstitial fluid through the osteocyte canalicular network from regions of high strain to low strain. Electrical potentials induced by the flow, along with the fluid shear stress are then thought to modulate the production of signalling factors such as IGF-1 and prostaglandins by the osteocytes[21]. It has also been suggested that osteocytes released from fracture sites may de-differentiate to osteoblasts, providing an urgently required source of bone forming cells at the trauma site [18].

Osteoclasts are giant multinucleated cells found at sites of bone turnover and are responsible for bone resorption[22-24]. These cells are of haemopoietic origin, derived from the CFU-M monocyte-macrophage family[25] (fig. 1. 5).
Progenitor cells are recruited from the haemopoietic tissues such as bone marrow and splenic tissues and transported to bone via the circulating bloodstream [26].

Osteoclasts are distinguished by their large size, acidophilic staining characteristics, foamy cytoplasms and, by their multinuclearity, commonly containing between 10-20 nuclei per cell. Other ultrastructural characteristics of the osteoclast include large numbers of lysosomes, numerous and pleomorphic mitochondria and extensive Golgi complexes located in the perinuclear area[27].

The portion of the cell directly in contact with the bone can be divided into two parts: a ruffled border containing numerous finger-like invaginations of the plasma membrane and a clear zone, an organelle free area rich in actin-like filaments. The clear zone demarcates the limit of bone area being resorbed (fig. 1. 6).

Osteoclasts resorb bone by the release of lysosomal hydrolases into the extracellular space at the ruffled border. Hydrolytic enzymes including collaganase, cause the digestion of the extracellular organic component of the extracellular matrix. Concomitantly, decalcification (i.e.. the dissolution of calcium salts) occurs through the secretion of organic acids such as carbonic acid, by the membranes of the ruffled border. Because acid hydrolases favour a low pH, the osteoclast maintains an acidic environment in the extracellular space between the bone and the osteoclast. It is thought that the ruffled border also provides a large surface area for the absorption of the degraded bone matrix[9,18]

Figure 1.5: Proposed Lineage of the Osteoclast [10].

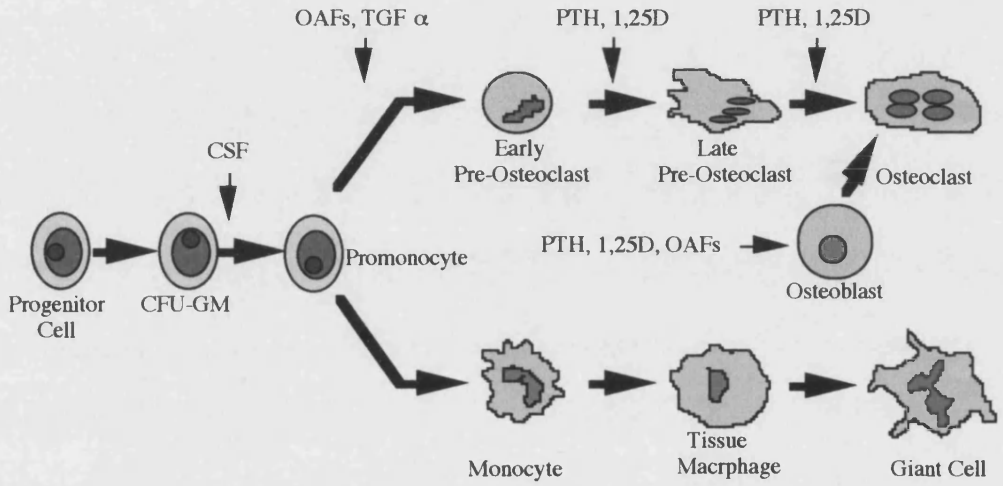
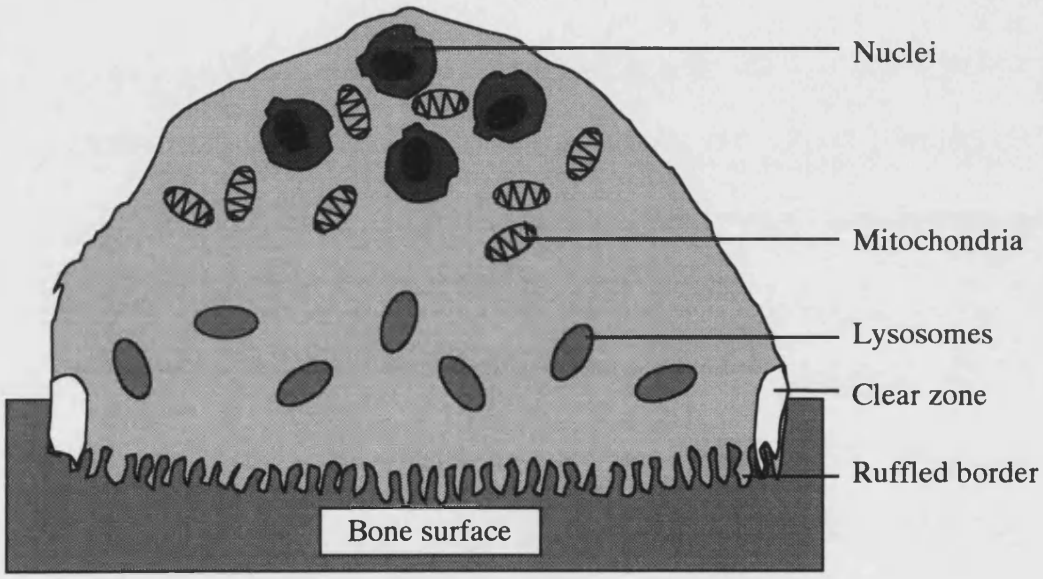


Figure 1.6: Schematic Diagram of an Osteoclast Showing Positions of Organells, Clear Zone and Ruffled Border



1.2.2: Bone Matrix.

The composition of bone matrix varies with bone type, site, infiltration of cartilage, marrow and blood vessels and therefore a complete and universal description is impossible28. Generally the bone matrix can be described as having an inorganic and an organic component at a dry weight ratio of 65:35 respectively.

The inorganic component comprises of calcium phosphate (85%) calcium carbonate (10%) with significant quantities of bicarbonate and hydroxyl ions. Magnesium, sodium, potassium, fluorite, citrate and sulphate are minor constituents. The calcium and phosphate form hydroxyapatite crystals of 1.5 x 30 nm dimensions. These bind to collagen in a parallel formation and are surrounded by osteoid18.

The organic component is mostly (approx. 90%) type I collagen, a fibrillar protein produced by osteoblasts. The type I collagen molecule is the basic building block of the bone matrix system. The molecule is a triple-helical supercoil containing two identical _1(I) chains and a structurally similar but genetically different _2(I) chain. In the matrix, individual collagen molecules are packed end to end with a short space between them. The molecules are packed laterally in a one quarter stagger array offsetting each molecule from its neighbour by one-fourth of its length10 (fig. 1.7).

This 3-dimensional arrangement constitutes the fibre structure found in the bone matrix. Minute amounts of type III, V and II, X may also be found but these are thought to be contaminants derived from blood vessels and cartilage respectively.

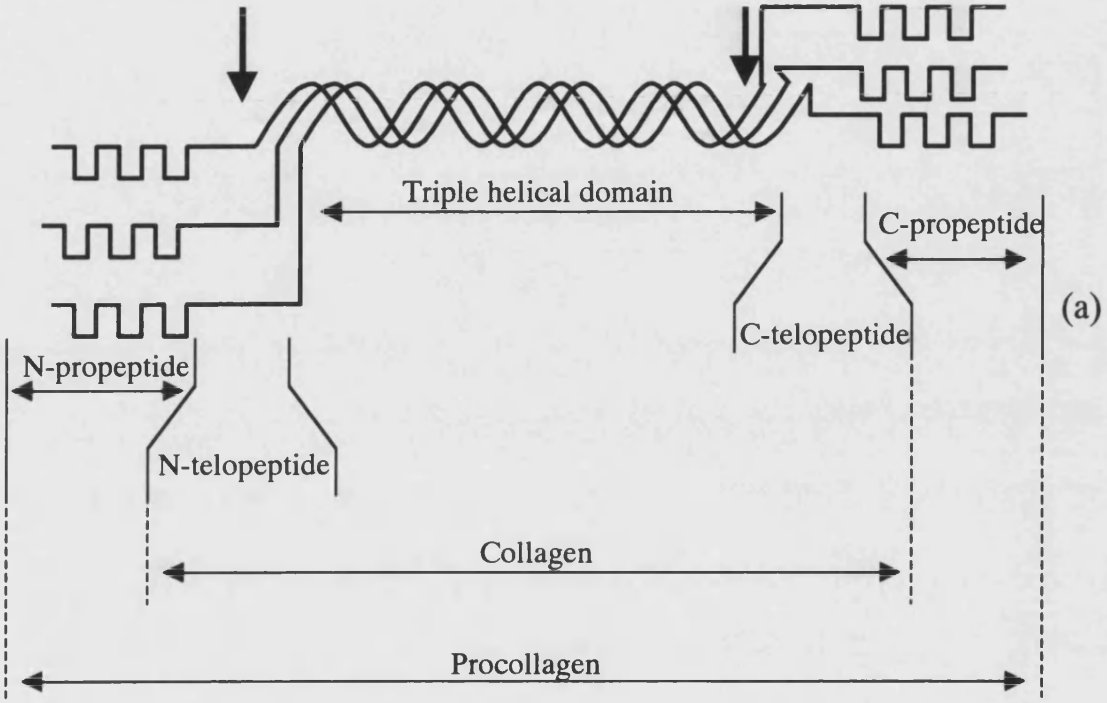
Non-collagenous proteins (NCP) present in the matrix comprise 10-15% of the total bone protein content and can be categorised into four general groups.10.

(i) Cell attachment proteins
(ii) Proteoglycans
(iii) γ-Carboxylated (gla) proteins
(iv) Growth related proteins

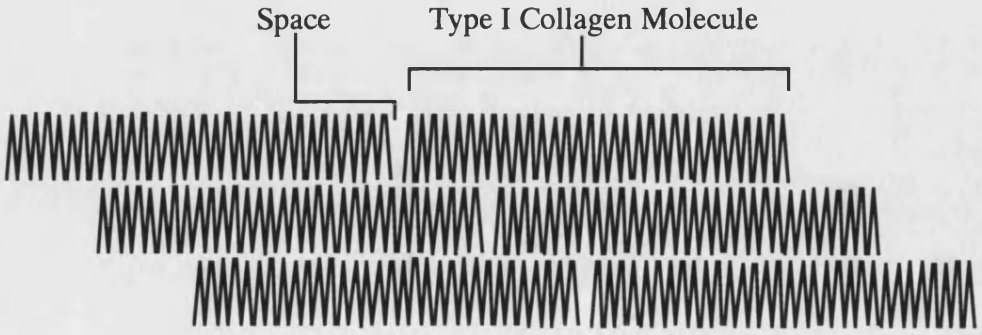
These classifications are often overlapping and by no means exhaustive as most of the roles for individual NCPs are currently undefined.

Figure 1.7: Type 1 collagen is secreted as a propeptide, but the N telopeptide and the C Telopeptide are rapidly cleaved by specific proteases (vertical arrows).
(adapted from Rossert, J., de Crombrugghe, B., Type I Collagen: Structure, Synthesis and Regulation. In; Principles in Bone Biology. Academic Press, San Diego 1996).

Figure 1.7: (a) Schematic Representation of a Type 1 Procollagen Molecule and (b) the Arrangement of the Collagen Molecule in Bone Matrix.



(a)



(b)

Cell attachment proteins. To facilitate bone cell interactions with the extracellular environment, bone cells synthesise four proteins that affect cell attachment: Fibronectin, thrombospondin, osteopontin and bone sialoprotein. Cell attachment is important for the co-ordination of many cellular functions including cell proliferation, differentiation and migration [29]. Osteopontin and bone sialoprotein are also known to anchor osteoclasts at resorption sites [30,31].

Proteoglycans are macromolecules that contain acidic polysaccharide side chains (glycosaminoglycans) attached to a central core protein. In bone two types of glycosaminoglycan are found: chondroitin sulphate and heparin sulphate [10]. Heparin sulfate proteoglycan is membrane associated and is thought to facilitate the interaction of osteoblasts with extracellular macromolecules such as fibronectin and thrombospondin as well as heparin binding growth factors [32]. The role of chondroitin sulfate is not yet understood but it is thought to play a role in preserving the integrity of the environment immediately outside the cell membrane. Most of the glycosaminoglycans of bone are attached to two small (40 kDa) proteoglycan core proteins, PG-I (biglycan) and PG-II (decorin), whose precise functions are not known but are assumed to be important for the integrity of most connective tissue matrices [33].

γ-Carboxylated (gla) proteins. Two structurally related, vitamin K-dependant, γ-carboxylated (gla) proteins are found among the NCPs: osteocalcin (bone gla-protein) and matrix gla-protein (MGP) [34]. Osteocalcin is bone specific and acts as a signal in the bone turnover cascade In knockout mice, bone mass was increased due to an increase in the rate of cortical and trabecular bone formation suggesting osteocalcin acts to inhibit bone formation *in vivo* [35]. The precise function of matrix gla-protein, which is found in cartilage and bone, is unknown [10].

Growth related proteins. The most abundant NCP produced by bone cells is the growth related protein osteonectin. This protein has high affinity for binding ionic calcium, [10] hydroxyapatite, collagen and thrombospondin [36]. It is also thought to participate in osteoblast growth and proliferation as well as matrix mineralisation.

Transforming Growth Factor β (TGFβ). TGFβ is a 390 amino acid (AA) polypeptide secreted by osteoblasts which belongs to a superfamily of multifunctional structurally related cytokines [37]. The importance of this molecule is revealed when it is realised that:

(i) TGFβ is highly conserved; identical in human, monkey, bovine, porcine and avian forms.

(ii) Many cell types produce TGFβ . (eg. Fibroblasts, mesothelial cells, transformed cells and osteoblasts)

(iii) Almost every cell type studied to date has receptors for TGFβ .

(iv) TGFβ has significant effects on almost every cell possessing receptors for it.

The TGFβ family includes three mammalian isoforms, TGFβ1,–β2, and–β3. Three types of receptor exist.; high affinity types I+II; and low affinity type III,[38] it is thought that most cells types possess these receptors[39]. Human TGFβ receptors bind all three isoforms; type I binds; β1 = β2 > β3; type II binds; β1 > β2 > β3and type III; β1 = β2 = β3 .

Receptor signalling. Receptors type I+II are serine/threonine specific protein kinases and signal transduction, upon ligand binding, requires the formation of a heteromeric complex of both (fig. 1.8). The type III receptors are thought not to transduce signals but function to concentrate TGFβ on the cell surface, presenting it to the Type I + II receptors[38].

Subsequent signal transduction has not been fully elucidated[40]. It is thought that the activation of the serine/threonine protein kinase domain of the type II subunit stimulates phospholipase C (PLC) breakdown of membrane inositol phospholipid; phosphatidylinositol 4,5 bisphosphate -> inositol 1,4,5 trisphosphate + diacyl glycerol (PIP_2 -> IP_3 + DAG). IP_3 stimulates the release of RER stored calcium, which along with DAG, activates a protein kinase C (PKC) induced calcium influx[38].

Recent work has implicated a number of potential downstream targets of the TGFβ superfamily pathways. The most convincing data in support of a critical role in this process are those obtained for the Smad related proteins (human homologue of mothers against decapentaplegic (MAD) gene from drosophila)[40-45].

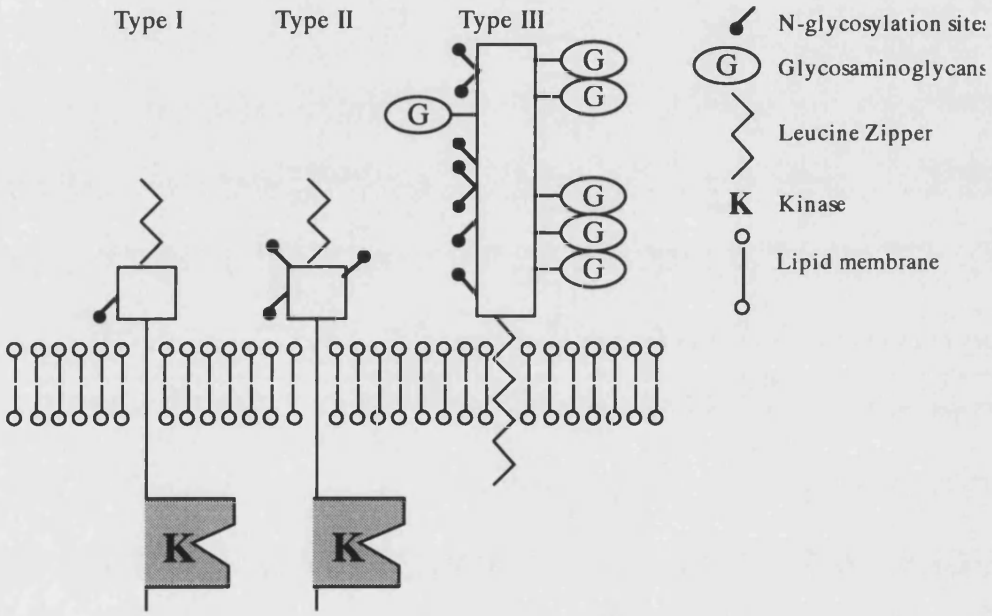
In mammalian cells, the induction of BMP signalling pathways causes the phosphorylation of Smad1 through some unknown pathway and, the subsequent translocation of the protein from the cytoplasm to the nucleous[46,47]. Although this suggests the possibility that Smads act as transcriptional regulators, it is not clear

whether the Smads bind directly to DNA or act via some intermediary DNA-binding proteins. (The PI second messenger system, overview fig. 1.18).

Biological Effects of TGFβ. TGFβ exerts a wide ranging autocrine and paracine regulatory influence on many cell types[39]. Studies have shown it to both stimulate and inhibit proliferation, modulate differentiation, angiogenesis, cell cycle progression, bone formation, and extracellular matrix production[48-52], all mechanisms important in tissue development, remodelling and repair. The diversity of effects modulated by TGFβ is dependant on phenotype and/or stage of differentiation[37].

In vitro, TGFβ has been shown to both inhibit and stimulate osteoblast cell proliferation and increase markers of osteoblast differentiation such as tissue non-specific alkaline phosphatase (AP), Type I collagen and osteonectin[53].
In vivo, TGFβ has been shown to regulate osteoblast differentiation and modulate the coupling of bone formation to bone resorption[54]. Treatment with TGFβcauses increased periosteal bone formation adjacent to sites of local application[53]. Fracture repair is enhanced by both the local[55,56] and systemic[57-59] application of TGFβ.

Figure 1.8: Schematic view of TGFβ Receptors[38]



Fibroblast Growth Factors Fibroblast Growth Factors (FGFs) were first characterised in the mid 1970s as a mitogen of cultured fibroblasts[60]. The FGF family currently has 9 members and their structural similarity suggest a common ancestral gene[61]. The biological effects of the FGFs are modulated via 3 discrete biochemical entities.

(i) Low affinity trans-membrane binding proteins
(ii) Heparin sulphate oligosaccharaides.
(iii) High affinity transmembrane tyrosine kinase receptors. [60].

To date, four tyrosine kinase receptors have been identified (FGFr 1-4)[38], where ligand binding activates intracellular signal transduction pathways. Activation requires receptor oligomerization, mediated by the association of heparin with the FGF ligand and, of the ligand with the receptor. The function of the low affinity transmembrane binding protein is unclear although suggestions indicating a role in the cellular uptake of FGF have been made[60].

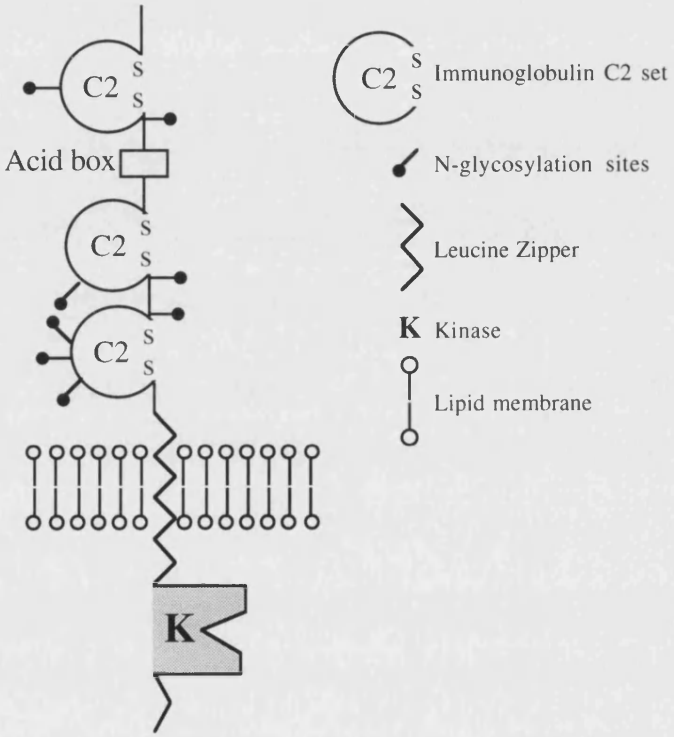
The four tyrosine kinase receptor proteins each contain an extracellular region containing three immunoglobulin (Ig) like domains, a transmembrane segment and a split cytoplasmic tyrosine kinase region[62] (fig. 1.9). The receptors have amino acid homology of 55-72% with differing ligand affinities and tissue distribution.

Splice variants of the FGF1-3 receptor genes give rise to multiple receptor forms which exhibit structural differences,- some of the receptor variants for example lack the first immunoglobulin domain while others exhibit differences in the carboxy terminal part of the 3rd immunoglobulin domain or, contain deletions within the intra or extracellular regions[63].

FGF receptors and their variants all bind more than one type of FGF with high affinity. Similarly, any given FGF can bind to more than one receptor with high affinity.

Receptor Signalling. Ligand binding to the FGFr stimulates dimerization and phosphorylation. Receptors then bind to PLCγ and stimulate the PI second messenger system. (Fig. 1.18).

Figure 1.9: Generalised FGF receptor [38].



Biological Effects of FGF. FGFs and their receptors play a crucial role in normal and abnormal bone development. This has been demonstrated recently with the discovery of unique mutations in FGFr 1-3 which give rise to a variety of skeletal disorders[62]. For example, a point mutation in the FGFr-3 gene causes achondroplasia, the most common form of dwarfism[64]. Similar defects in the FGFr-2 and FGFr-1 genes cause Crouzon and Pfeiffer Syndromes respectively, both examples of craniosyntosis disorders [62].

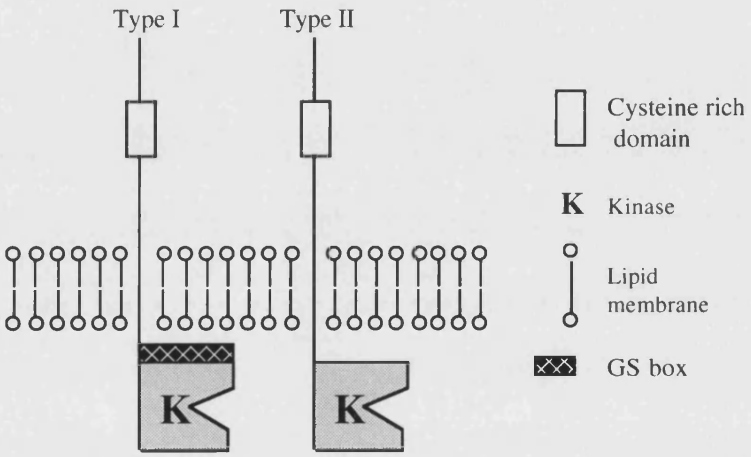
In vitro, FGF-2 has been shown to be a potent mitogen for bone marrow stromal cells and to increase the capacity of these cells to produce a bone-like tissue[65-67].
In vivo, treatment with FGF-2 stimulates osteoblast growth[68] and increases bone formation[69-71] enhancing fracture repair [72].

Bone Morphogenetic Proteins (BMPs). BMPs are dimeric growth factors which comprise part of the TGFβ superfamily of multifunctional structurally related cytokines. Nine BMPs have so far been identified, all having relative degrees of sequence homology and exhibiting a wide range of biological functions in a diversity of tissue types[73]. Common to most BMPs is the ability to induce osteogenesis in bone and at ectopic sites[74-76].

Although much less information exists regarding the BMP receptor species than is known for other members of the TGFβ family, it is known that the BMP osteoinductive mechanism is modulated via two distinct receptors of the serine/threonine kinase family[77](fig. 1.10). Type I receptors are subdivided into subtypes ALK-2, ALK-3, and ALK-6 while Type II receptors are further divided into T-ALK, ActRII and ActRIIb subtypes. The Type I+II receptors have been shown to bind only BMPs 2, 4, and 7, receptors for other BMPs are not apparent at the present time.

Unlike receptors for TGFβ, BMP Type I and II receptors can bind ligands in the absence of each other, although formation of a Type I-II receptor complex may be a requirement for downstream signalling[78]. The precise downstream signalling pathway, initiated by BMP ligand binding, is currently not well understood[79].

Figure 1.10: Schematic diagram of BMP receptors.



Biological Effects of BMPs. *In vivo* , implantation of BMPs-2, 4, 5, 6, 7 or 9 into a wide range of tissues induces bone and cartilage formation[75,80-82]. Interestingly, all the heterodimeric BMPs tested to date have demonstrated a higher specific activity than their homodimer components[79].

In vitro, BMPs have demonstrated an ability to act as both positive and negative regulators of proliferation and differentiation in a number of cell types[83]. Their most striking characteristic is their ability to induce an osteogenic phenotype in undifferentiated mesenchymal cells[79,84].

Taken as a whole, current knowledge strongly suggests that the primary target of BMPs is an early mesenchymal cell which when challenged with BMPs differentiates into osteoblast-like and chondroblast-like cells.

Insulin-like Growth Factors (IGF-I, IGF-II). The IGFs are anabolic peptides structurally and functionally related to insulin and are the most abundant of the growth factors located within the bone microenvironment[85]. An important function of the IGFs is to act as paracine/autocrine regulators of bone formation[86].

IGFs exist *in vivo* complexed to larger proteins termed IGF binding proteins (IGFBPs). Six IGFBPs are known to exist, IGFBP 1-6, and these function to prolong the half-life of the small IGF molecule and, because most IGFBPs prevent IGF binding to IGF receptors, they also act as regulators of IGF activity[87].

IGFs exert their effects via two specific membrane spanning receptors[88,89]. The Type I is a tyrosine kinase receptor which is structurally related to the insulin receptor with a preferential binding affinity: IGF-I > IGF-II > Insulin. Ligand binding to the Type I receptor initiates the PI second messenger system. The Type II receptor binds IGF-II and lysosomal enzymes with high affinity but does not bind insulin[90], it is also lacking in tyrosine kinase activity. It is thought the Type II receptor is involved in receptor mediated internalisation and lysosomal enzyme sorting and trafficking[91].

Biological Effects of IGFs. As with other bone derived growth factors, IGFs not only act as acute paracine/autocrine regulators, but also become incorporated into bone matrix and may be released later during resorption. IGFs modulate bone formation in a number of ways; by stimulating proliferation and differentiation of osteoblast precursors[92]; by modulating the effects of systemic hormones on bone formation[92]; and acting as a paracrine regulator of osteoclast resorption[93]. IGF infusion increases bone formation in normal and osteopenic rats[94] and significantly improves bone repair in calvarial defects[95]. *In vitro* IGFs increase deoxyribonucleic acid (DNA) synthesis and replication of cells of the osteoblast lineage and stimulate differentiated function of the mature osteoblast[86,96].

Platelet Derived Growth Factor (PDGF). PDGF is a polypeptide growth factor that was initially isolated from human platelets and subsequently found in other cell systems. Since PDGF is present in platelets and expressed in other cell types it is considered to act as both a systemic and local regulator of cell metabolism[97].

PDGF is composed of two polypeptide chains that may be combined in a homo- or heterodimeric form. The chains are products of the related genes PDGF A and PDGF B, so PDGF can exist as either PDGF AA, PDGF BB homodimers or PDGF AB heterodimer[98].

Two PDGF receptors are recognised, α and β, (fig. 1.11) which have a 44% amino acid sequence homology. The receptors are members of the tyrosine kinase family, containing a catalytic tyrosine kinase domain in the intracellular region of the receptor and a immunoglobulin-like domains in the extracellular portion [99]. PDGF A chains bind primarily to the α receptor whereas PDGF B chains bind to either the α or β receptor [100]. PDGF binding induces dimerization and activates downstream signal transduction pathways which are not well established. It is thought that the PKC, -phospholipase C-γ , -PI- 3 kinase,- calcium flux pathway is involved[101].

PDGF AA, BB and AB have similar biological actions in skeletal and non-skeletal cells but with differing potencies. The efficacy of the different isoforms is ranked thus: BB > AB > AA[102]. The primary effect of PDGF on bone is mitogenic, stimulating bone cell replication in both intact calvaria and isolated osteoblasts[102]. Exposure to PDGF does not enhance the differentiation of the osteoblast and it is thought that its role may be to increase the pool of osteoprogenitor cells which will eventually express the osteoblast phenotype[97]. In addition to its effects on bone cell

proliferation, PDGF BB and AB increase bone resorption by a mechanism which is currently unknown [103].

Considered as a whole, current knowledge suggests that it is unlikely that PDGF A or B, play a role in the maintenance of bone mass, however the actions of PDGF on bone cell replication suggest a possible role in fracture healing and repair.

Figure 1.11: Generalised PDGF receptor.

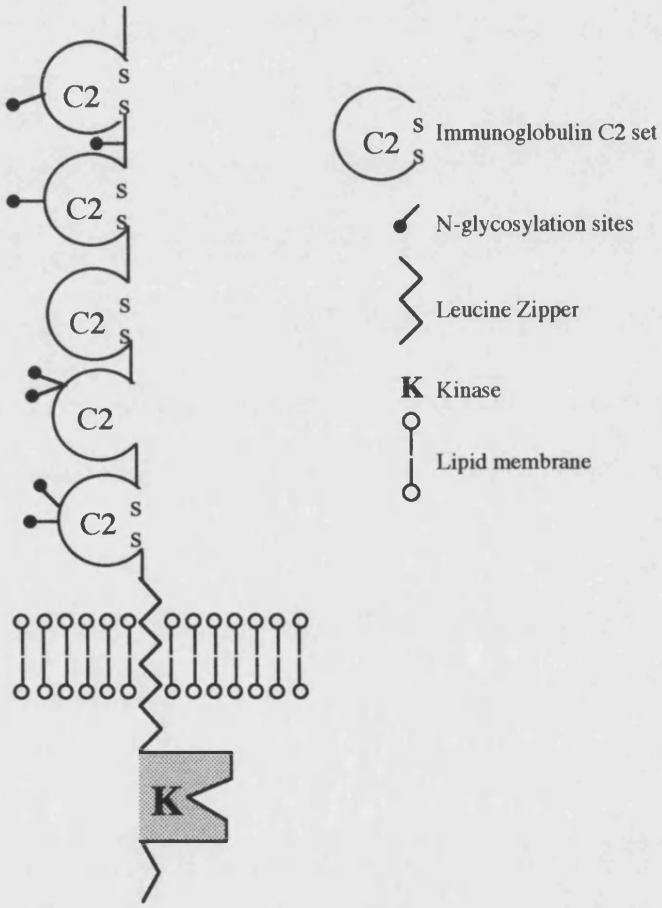


Table 1.2: Summary of Bone Cell Derived Growth Factors and their Effects on Osteoblasts[104,105].

Factor	kDa	Concentration in matrix (ng/g)	Proliferation	Differentiation
IGF2	7.5	1500	↑	↑
TGFβ	50	450	↑	↑
IGF1	7.7	100	↑	↑
PDGF	35	60	↑	↓
FGF	33	60	↑	↓
BMP-2	30-35	Total, 1-2	↑	↑
BMP-3	30-40		↑	↑
BMP-4	30-35		↑	↑
BMP-5	30-35		↑	?
BMP-6	30-35		↑	?
BMP-7	30-35		↑	↑
BMP-8	30-35		↑	↑
BMP-9	30-35		↑	↑

1.2.3: Organisation of Bone.

The cells and matrix of bone tissue are organised to maximise the relationship between structure and function. Bone which forms initially in development (i.e.. primitive bone) is termed woven bone and this is transformed through remodelling processes into lamellar bone.

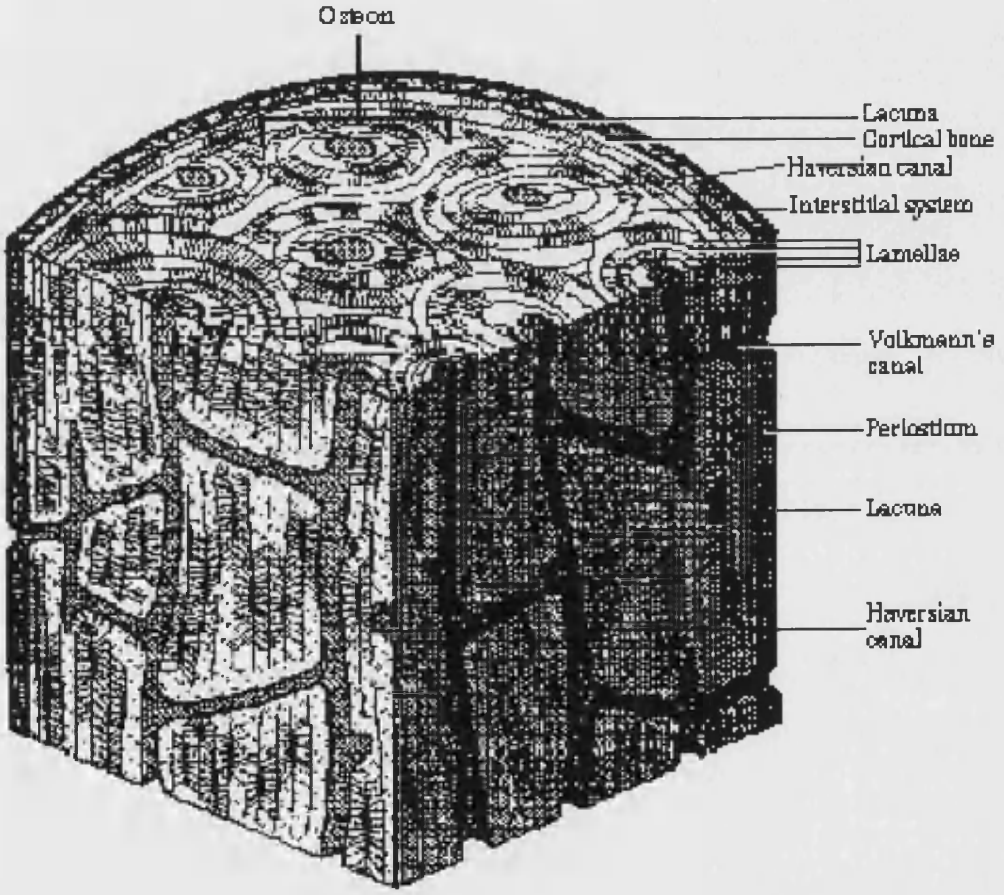
Bone may be classified in a number of ways and can be characterised as cortical (compact) or trabecular (cancellous or spongy). Cortical and trabecular bone are constituted of the same cells and matrix elements but there are structural and functional differences[106]. Trabecular bone like that found inside the epiphysis, or head of long bones is always surrounded by cortical bone. Trabecular bone has large, open spaces surrounded by thin, anastomosing plates of bone. Cortical bone is much denser than trabecular.

In cortical bone of the human the smallest organisational unit is the osteon, or Haversian system (fig. 1.12). This structure consists of a central Haversian canal surrounded by concentric lamellae (of mineralised matrix) in which are found osteocytes residing in cavities known as lacunae. The function of the Haversian canal is to provide a cavity through which arteries, veins and nerves may pass.[107].

Osteocytes intercommunicate by utilising their long cytoplasmic extensions via canaliculi, small communicating channels found between the lacunae. Running at right angles to the Haversian canals are found Volkmann's canals, these provide extra pathways, allowing a greater mass of bone to be reached by the neuro-vascular system. The mass of dense compact bone is formed by parallel arrangements of osteons and this arrangement encloses the alternative type of bone mentioned above, the cancellous bone[9].

Similar to compact bone, cancellous bone possesses lacunae housing osteocytes, but unlike compact bone, the matrix is not arranged into osteons. Cancellous bone is comprised of a lattice of matrix, giving the appearance of it being sponge-like to the naked eye. This arrangement of matrix allows an efficient distribution of applied loads.

Figure 1.12: Cortical Bone Organisation.



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Three distinct surfaces, or envelopes, of bone are recognised[108] the periosteum, the endosteum and the Haversian surface. Except for the articular surfaces, the external surface of the bone is surrounded by the periosteum, a two layered membrane with an outer fibrous collagenous layer and an inner osteogenic layer. In this inner layer are found osteogenic cells and osteoblasts, both cell types of fundamental importance in bone remodelling. The Haversian surfaces are found lining the Haversian canals and the remaining bone substance of the cancellous trabeculum is enclosed in endosteum. The periosteum is attached to bone via Sharpeys fibres,- collagenous bundles of fibrocartilage trapped in the calcified bone matrix[108].

Bone formation. To understand this complex process it is important to distinguish between the development of bone as an organ and the histogenesis of bone tissue. The development of bone is traditionally classified as either endochondral or intramembranous ossification[18].

Endochondral ossification. In this process, proliferation and aggregation of mesenchymal cells occur at the site of impending bone formation. The mesenchymal cells differentiate into chondroblasts which produce a hyaline cartilage matrix acquiring the general shape and appearance of the specific bone to be formed. Ossification occurs when the perichondrium (a cartilage equivalent to bone periosteum) ceases to give rise to chondrocytes and instead produces osteoblasts. Because of the change of function the perichondrium is now called the periosteum. As a result of the changes a thin layer of bone is formed around the cartilage template. With the establishment of the periosteal bone, chondrocytes in the cartilage become hypertrophic resulting in the compression of the surrounding cartilage. The compressed cartilage forms irregular plates between the hypertrophic cells which at this stage express alkaline phosphatase causing the concomitant calcification of the cartilage matrix[9].

The calcification of the matrix inhibits the diffusion of nutrients resulting in the death of chondrocytes and the breakdown of the matrix. In the case of a long bone this cavity eventually becomes the marrow space. At this stage, blood vessels invade and vascularise the cavity providing an access to the primitive cells which eventually give rise to the marrow.

Intramembranous ossification. In this mechanism bone is formed through the differentiation of mesenchymal cells into osteoblasts. This process is initiated when condensations of mesenchymal cells within mesenchymal tissue congregate at sites

destined to become bone. (The condensations of cells in the tissue is the "membrane" referred to in the term "intramembranous ossification"). As the process continues, the newly organised tissue becomes more vascularised, this initiates the osteogenic differentiation of the mesenchymal cells. The newly formed osteoblasts secrete osteoid which in time becomes calcified, with a proportion of the osteoblasts becoming trapped in the osteoid as described above. As osteoblasts are non-dividing cells they are replaced by the proliferation of primitive cells from the "membrane" which eventually give rise to osteoprogenitor cell[9].

1.3: Bone Remodelling.

The bone remodelling cycle is a complex process co-ordinated by an elaborate interplay between systemic hormones, local bone derived factors and cytokines.
This process is achieved through the actions of basic multicellular units (BMUs), teams of bone resorbing osteoclasts and bone building osteoblasts[109]. BMUs engineer the resorption of old bone by osteoclasts and the subsequent deposition of new bone by osteoblasts (fig. 1.13). This allows the skeleton to respond to microdamage by continuous modification through renewal and repair, whilst maintaining its anatomical and structural integrity. The role of the skeleton as a mineral reservoir is also facilitated by the remodelling process. The normal sequence of bone remodelling is outlined below:[4]

(i) Quiescence: Inactive bone surface covered in lining cells

(ii) Recruitment: Mononuclear osteoclast precursors are recruited locally

(iii) Activation: Bone lining cells respond to bone resorbing hormones by contracting to allow osteoclast access and release of proteolytic enzymes (collaganase, plasminogen activators)

(iv) Resorption: As osteoclasts differentiate, migrate and fuse with the bone surface. Resorption occurs as osteoclasts release mineral dissolving hydrogen ions and matrix dissolving lysosomal enzymes. Osteoblast stimulating growth factors are released and activated as a by product of this process. When resorption is complete, the reversal phase occurs. Macrophages-like cells appear on the surface and complete resorption and/or, produce factors which aid osteoblastic bone formation. However, their exact role is uncertain.

(v) Formation: New bone replacement depends on four main factors and a departure from these conditions may lead to a pathological imbalance in the bone resorption / formation cycle[6]. The factors are:

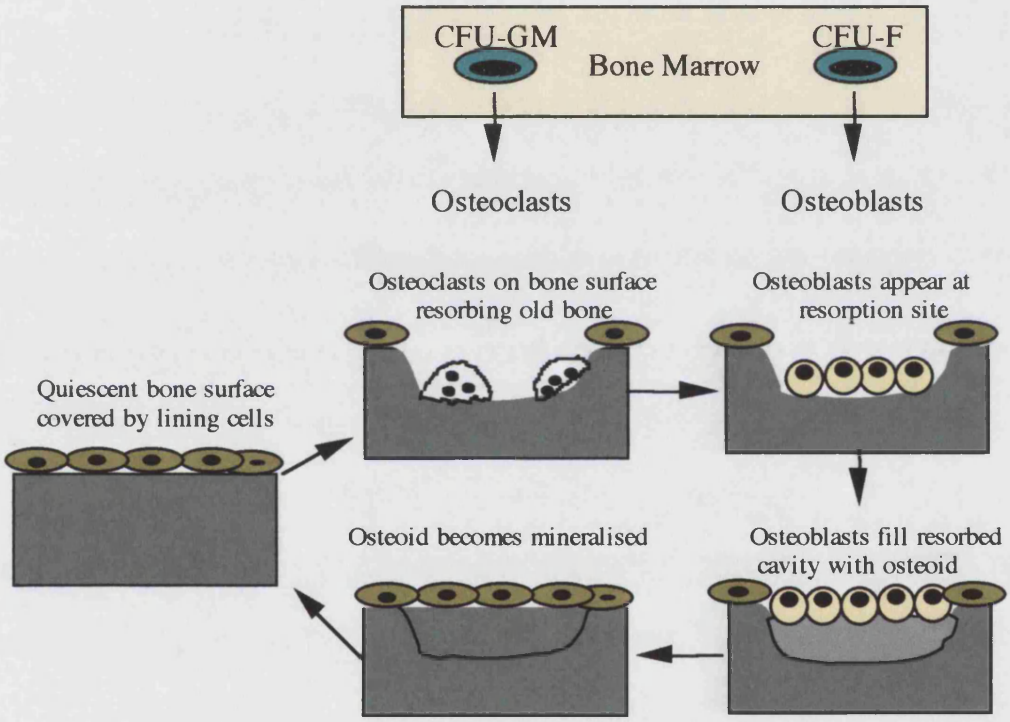
(i) Presence of unresorbed bone to act as a template

(ii) Release of local growth factors from cells and matrix which provide the signal for osteoblast activation.

(iii) Ability of osteoblast precursors to proliferate and differentiate

(iv) Capacity of the osteoblast to produce normal matrix.

Figure 1.13: The Bone Remodelling Process (adapted from Manolagas [4]).



The systemic hormones that are involved with the regulation of remodelling can be subdivided into three main categories: polypeptides, steroids and thyroid hormones, (Table 1. 3)

Table 1. 3

Systemic Hormones

Polypeptides	Steroid	Thyroid
Parathyroid hormone	1, 25-Dihydroxyvitamin D3	Thyroid hormone
Calcitonin	Glucocorticoids	Calcitonin
Insulin	Estrogens	
Growth hormone		

An overview of these hormones (below) will place emphasis on those having particular relevance to this study.

1.3.1: Parathyroid Hormone (PTH), Calcitonin and 1, 25-Dihydroxyvitamin D_3.

PTH is an 84 amino acid polypeptide secreted by the parathyroid gland, calcitonin a 32 amino acid polypeptide secreted by the thyroid gland and 1, 25-dihydroxyvitamin D3 (calcitriol), a steroid hormone synthesised primarily in the kidney. The principal function of these three hormones is to act in concert to regulate serum calcium and phosphate levels[10] (fig. 1.14). Physiologically, their target organs are the kidney, the intestine and bone. Regulation is achieved by four mechanisms:

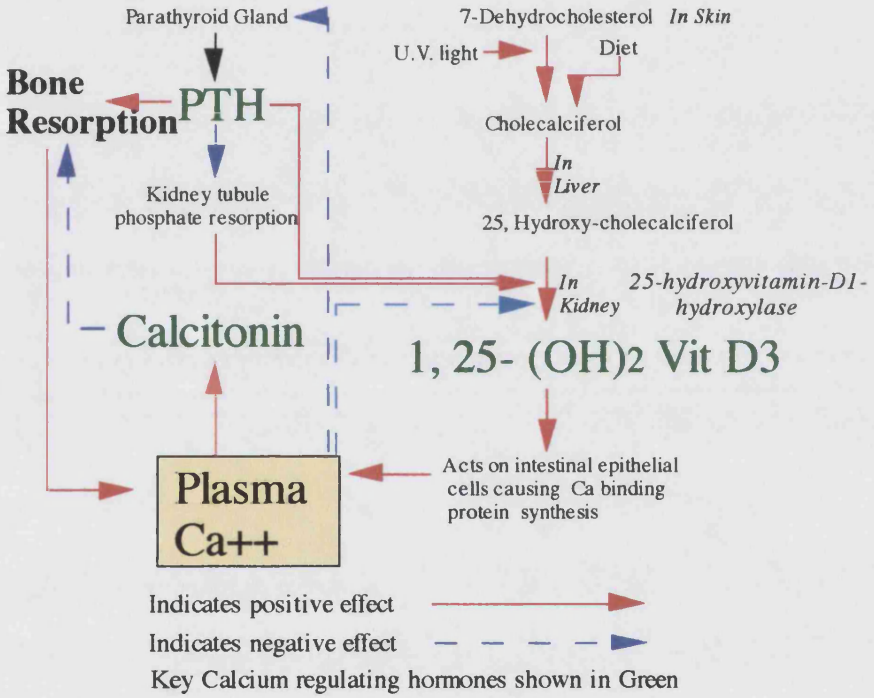
(i) PTH stimulates calcium reabsorption and phosphate excretion from the glomerular filtrate. Phosphate is normally reabsorbed from the glomerular filtrate in both the proximal and distal tubules, PTH acting on these sites, inhibits phosphate absorption. PTH treatment also decreases calcium reabsorbtion in the proximal tubule but this loss is compensated by increased absorption in the distal tubule, causing a net gain in serum calcium levels[110].

(ii) PTH stimulation of the renal conversion of 25 $(OH)_2 D_3$ ->1, $25(OH)_2 D_3$ thereby increasing the absorption of calcium and phosphate across the gut[111].

(iii) PTH stimulation of calcium and phosphate release from bone[10]. PTH actions on bone are somewhat paradoxical and poorly understood. In its familiar physiological role, chronic administration of PTH stimulates osteoclastogenesis and hence bone resorption[10,112,113]

Figure 1.14: Schematic representation of calcium homeostasis regulated by PTH, calcitonin and 1, 25- (OH)2 Vit D3 interaction.

Figure 1.14: Overview of Calcium Regulation.



(iv) Calcitonin inhibition of bone resorption by directly inhibiting the activity of the mature osteoclast.

Although some investigators have proposed that PTH has a direct effect on osteoclasts [114,115] the conventional hypothesis considers it to be an indirect action. PTH is thought to act indirectly on osteoclasts by either stimulating osteoblasts[116], stromal cells[117,118] or other bone marrow cell types (e.g. mast cells), to secrete paracrine factors, such as interleukin-6 (IL-6) and IL-11 [119-121], which act locally. Alternatively, it may modulate cell-cell interactions between osteoblasts and osteoclasts[25,122]. Osteoclast access to bone surfaces is enhanced by PTH induced retraction of bone lining cells from resorption sites[123]

The potential anabolic actions of PTH were first recognised in 1922, when a study revealed that PTH extract could increase bone density in the young rat [124]. Since then PTH has been shown to enhance bone formation in animal models[15,125-134] as well as in the human[135-138]. PTH induced bone formation increases bone strength[132,139,140] without altering geometry and involves both trabecular[125,131,141,142] and cortical bone[125,143].

The process of PTH induced bone formation has been attributed to increased osteoblast proliferation and activity associated with increased levels of osteocalcin and BMP gene expression[144-146] the activation and differentiation of bone lining cells to osteoblast phenotype[15,16] and the stimulation of the proliferation and differentiation of osteoprogenitor cells in the bone marrow [126]. *In vitro* evidence suggests that the PTH induced bone formation is mediated by local production of IGF-I [129,147,148] or TGFβ [129,149,150].

PTH mediates its effects through interaction with a receptor linked to a guanine nucleotide binding protein (G (s) protein(GDP))[151] (fig. 1.15). In the resting state GDP is bound to the α subunit of the protein, PTH binding induces a conformational change in the receptor causing it to bind to the G-protein. The activated G-Protein releases GDP and binds GTP (Guanine triphosphate) initiating the dissociation of the α, β and γ subunits. The free α subunit binds adenylate cyclase (AC), catalysing the synthesis of cyclic adenosine-5'-monophosphate (cAMP) from adenosine-5'-triphosphate (ATP)[146,152,153]. cAMP dependant protein kinase, protein kinase A, (PKA) then phosphorylates gene regulating proteins.

Depending on the pulse amplitude and frequency modulation of the applied PTH an additional signalling pathway may be activated. Binding of PTH can activate a G(p) protein coupled to PLC[154] resulting in the hydrolysis of PIP2 to IP3 and DAG.[155]. The production of IP3 leads to the release of intracellular calcium stores, and DAG activates PKC, inducing C-FOS gene expression [122,156-158]. See figure 1.18 for signalling overview.

Previously it was thought that two separate PTH receptor classes were responsible for the activation of these separate pathways[159] but more recent evidence from binding studies indicate only one class of PTH receptor is responsible[154]. It is now accepted that the differential modulation of the PTH stimulated PKA and PKC pathways[125,160] accounts for the opposing effects seen with different PTH treatment regimes.

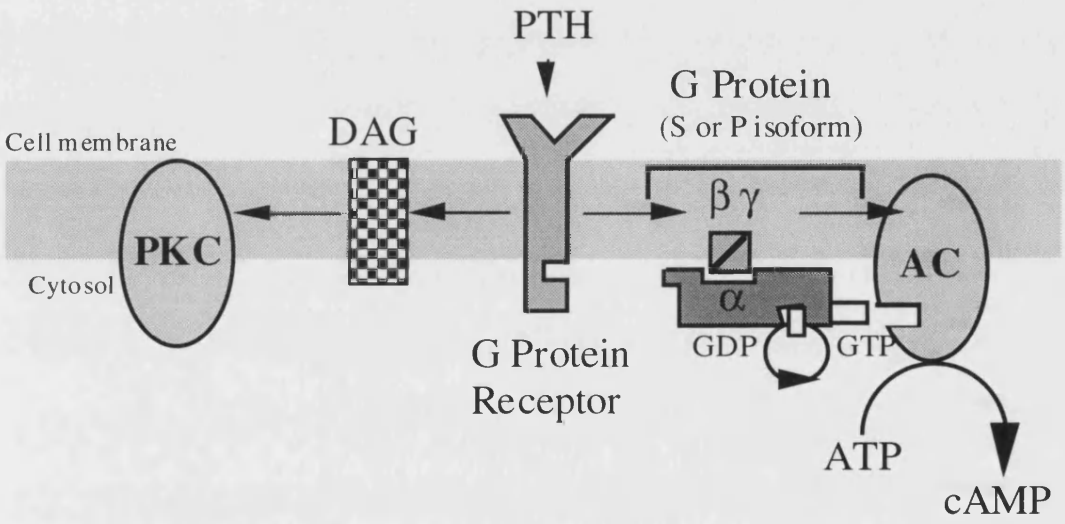
PTH stimulation of bone formation is predominantly mediated via the PKA pathway [109,125,142,144,161] although it is suggested a lesser involvement from the PKC pathway may also be required[144,161]. Bone resorption however is modulated chiefly by the PKC pathway [119,162] with some contribution from the PKA pathway[112].

The ability of the PTHr to activate multiple signalling pathways depends, at least in part, on the presence of different functional domains in the PTH molecule. The resulting structure : function studies can be summarised as follows:

The C terminal fragments are biologically inactive[163]. Fragment AA1-7 activates PKA pathway when some part of the 25-34 region is also present[109,164]. Region AA1-31 stimulates PKA only[109,141,144,165] and AA28-33 stimulates PKC and mitogenic activity[109,164]. Both the PKA and PKC pathways are induced by PTH fragment AA1-34 and the intact hormone AA1-84[109,125,144]. The anabolic effect of PTH is diminished by removal of AA1 and abolished by removal of AAs 1+2 [109,164].

Intracellularly, activation of the PKA pathway is associated with bone formation while activation of the PKC path is related to resorption. Accepting that activation of the PKA and PKC pathways are associated with particular domains of the PTH molecule it can be deduced that a relationship exists between PTH treatments and the PTH domain which binds the receptor. The mechanics of this correlation are not understood.

Figure 1.15: Generalised PTH G-Protein Receptor.



As can be seen, the effect of PTH on bone cell activity is wide ranging and somewhat contradictory, although it is widely accepted that the osteogenic effects of PTH are related to intermittent treatments and the osteolytic effects with chronic applications. These findings have indicated that a PTH fragment could eventually become the basis of novel treatments for osteoporosis and other conditions of bone deficit, and, consequently has stimulated research interest in that area [166,167].

1.3.2: Other Hormones Influencing Bone Turnover.

The naturally occurring corticosteroid, cortisol is produced in the adrenal cortex. Synthetic derivatives of cortisol, (e.g. dexamethasone (Dx), prednisone, prednisilone) have potent anti-inflammatory and immunosuppressant effects and are commonly used in the treatment of asthma, cardio-vascular disease and inflammatory bowel disease [168].

Glucocorticoids exert significant effects on bone metabolism via classical glucocoricoid receptors [169,170]. Unlike other hormones (except thyroid) steroids pass through the cell membrane by diffusion and bind to specific receptor proteins in the nucleus. The hormone receptor complex (HRC) then binds to specific DNA sequences called Hormone Responsive Elements (HREs). The HREs then either enhance or suppress the expression of specific genes adjacent to the HREs.

In vivo glucocorticoids stimulate bone resorption and inhibit formation leading to an overall bone deficit [171]. Patients subjected to long term treatment with glucocorticoid suffer significant bone loss [172-174]. The mechanisms by which glucocorticoids modulate these effects are complex and beyond the scope of this review. Briefly, glucocorticoids affect nearly every system in the body and those systems that modulate bone remodelling are overviewed in table 1. 4 [168].

Table 1.4.

Effects of Glucocorticoids on Systems that Modulate Bone Remodelling.

Pituitary
- Inhibition of secretion of:
  - Growth Hormone
  - Follicle Stimulating Hormone/Luteinising Hormone
  - Adrenocorticosteroid Hormone

Cellular Transport
- Decrease in transport of:
  - Calcium
  - Phosphorous

Parathyroid hormone
- Increased secretion
- Increased peripheral sensitivity to PTH

Gonads
- Inhibition of the synthesis of:
  - Estrogen by ovary
  - Testosterone by testes

Adrenal
- Decrease in secretion (due to suppression of ACTH) of:
  - Dehydroepiandrosterone
  - Androstenedione

In vitro, glucocorticoids may either stimulate of inhibit bone formation. In order to understand this paradox it is important to distinguish between the development and the regulatory effect of glucocorticoids on bone metabolism.

It is suggested that glucocorticoids first cause the proliferation and differentiation of a distinct population of osteoprogenitors cells that participate in bone formation [175-180] then in more differentiated cells, limit further proliferation [181]. It can be seen therefore that the effects of glucocorticoids in culture systems are dependant on the timing of the addition of the hormone coupled to the stage differentiation of the cells.

Glucocorticoid effects on bone metabolism can be direct or indirect. Directly, glucocorticoids modify the expression of C-Fos and C-Jun whose relative ratios regulate the expression of other genes [171], as well as the expression of osteoblast specific genes such as osteocalcin and collagen Type I.

Indirectly, glucocorticoid effects may be due to the modification in the synthesis of activity of growth factors secreted by bone. IGF-1 and TGFβ are important bone modulating growth factors whose expression and activity are affected by glucocorticoid treatment. Glucocorticoid treatment stimulates osteoblasts to activate

latent TGFβ[182] and decrease its mitogenic properties in bone cells[183] The activation of TGF β by glucocorticoids in bone cells may be relevant to the actions of the steroid on bone resorption.

The expression of IGF-I but not IGF-II, is directly down regulated by glucocorticoids [184] and it is likely that the inhibitory action of steroids on IGF-I synthesis is related to its effects on bone formation. The effects of glucocorticoids on other important bone modulating factors, the FGFs, BMPs and PDGF, are currently unknown 503}.

Insulin. Insulin is a polypeptide hormone synthesised by the β cells of the pancreas. Its main effect on bone remodelling is the stimulation of bone matrix synthesis and cartilage formation without affecting cell proliferation. This suggests that its effect is on the differentiated function of the osteoblast. Although insulin has direct stimulatory effects on bone tissue it also increases IGF production by the liver, therefore, some of the *in vivo* effects of insulin may be mediated by IGF. Insulin has no effect on bone resorption [10]

Growth hormone Growth hormone is a pituitary polypeptide which does not have a direct effect on bone resorption but, is thought to affect bone formation by direct stimulation of osteoblast activity and indirect stimulation of IGF synthesis. Growth hormone is essential for the maintenance of normal bone mass[185].

Estrogens and androgens. Estrogens and androgens are important for the prevention of bone loss and the maturation of the skeleton. The anti-resorption effect estrogen is thought to be mediated via the down regulation of cytokines such as interleukin-6, a potent resorption promoter[186].

Thyroid hormone. Thyroid hormones have no effect on bone matrix synthesis or osteoblast proliferation but are known to stimulate resorption. They also play an essential role in cartilage formation and are therefore important for normal development and growth[10].

1.3.3: Cytokines.

A number of cytokines which exert important effects in the immune and haematopoietic systems are produced by, and also influence the activities of skeletal cells. These include IL-1, IL-6, macrophage and granulocuyte/macrophage colony stimulating factors and tumour necrosis factor (TNF).

IL-1 has complex effects on bone remodelling and stimulates both bone cell replication and bone resorption[187]. IL-1 also increases the synthesis of IL-6 which increases bone resorption through the recruitment of cells in the osteoclast lineage.

IL-6 synthesis is inhibited by estrogens and this effect could explain the decrease in bone resorption following estrogen therapy[188]. TNF-α stimulates bone resorption, bone cell replication and inhibits osteoblastic differentiation[189]. Colony stimulating factors play a role in the maturation of osteoclasts, and granulocyte/macrophage-colony stimulating factor secretion is stimulated by PTH.

1.4: *In Vitro* Models of Differentiation.

As bone retains the capability to remodel, reform and repair throughout life, a reservoir of progenitors for bone forming and resorbing cells is required. An investigation of the factors that regulate this stem cell pool and induce commitment to, and expansion of, the osteogenic, or the adipogenic population may lead to an understanding of osteoblast decline in osteoporosis.

It is well established these osteogenic precursors exist in association with the soft connective tissue of the marrow stroma[190] and it is proposed that these precursors are commonly shared by cells of other types[191].

A popular thesis proposes that adipogenic, reticular and fibroblastic cell lines are all derived from the same progenitor cell type as osteogenic cells (fig. 1.16).

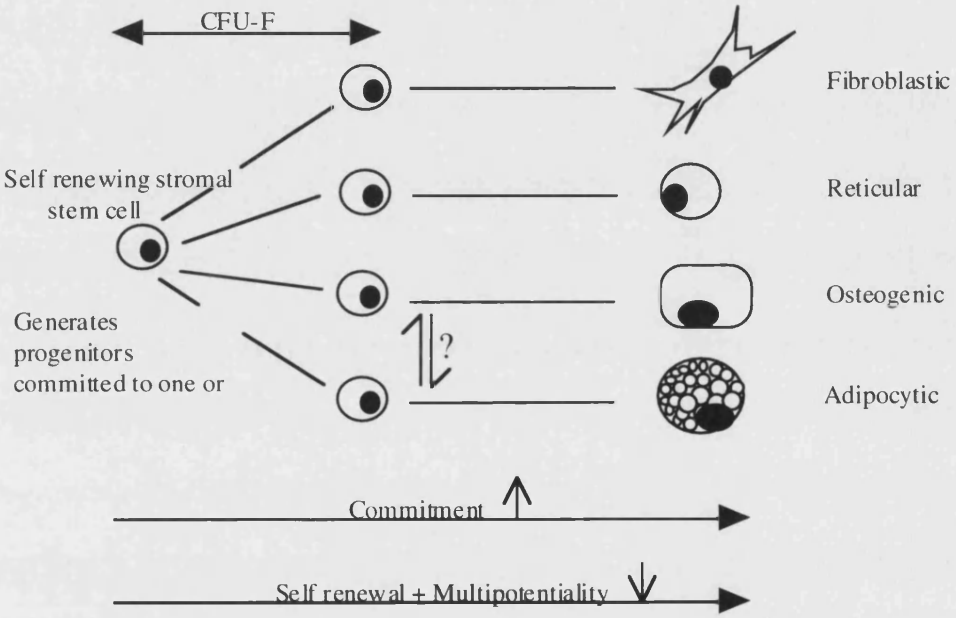
Numerous studies have demonstrated that when suspensions of bone marrow cells are plated *in vitro,* clonal colonies of fibroblastic shaped cells are formed[169,190-194]. Each colony is derived from a single (self-renewing) initiating a cell type termed a Colony Forming Unit Fibroblastic (CFU-F) [195]. Importantly, a proportion of these cells when transplanted *in vivo* , exhibit the characteristics of stem cells[196]. It is thought that CFU-Fs with high proliferative capacity represent the primitive stem cell[197] while colonies with lesser capacity represent cells at later stages of differentiation[198].

Presently, knowledge of the commitment steps from early stem cells, to committed osteoprogenitor, through to pre-osteoblast and osteoblast is relatively weak[11]. An understanding of the markers associated with the different stages of osteoblast differentiation will provide new insights into the nature of the osteoblast and its lineage relationship. Figure 1.17 represents an overview of the osteoblastic stage specific markers utilised in this study and are summarised below.

1.4.1: Osteoblastic Markers.

Alkaline phosphatase (AP). (orthophosphoric-monoester phosphohydrolase, alkaline optimum) is a protein ubiquitous in nature whose exact physiological functions are unknown[199]. The enzyme is covalently bound to phosphatidyl inositol (PI) phospholipid complexes in the plasma membrane where it functions to hydrolyse monophosphate esters at a high pH[12].

Figure 1.16: Proposed Marrow Stromal Cell Lineages. (Adapted from Owen [191]).



In the human, three tissue associated alkaline phosphatase isoenzymes exist: intestinal, placental, and germ cell (placental-like), along with a fourth more widely distributed isoform known as bone/liver/kidney alkaline phosphatase or tissue-non-specific alkaline phosphatase (AP)[200]. The four isoenzymes are encoded by four distinct genes[200], the bone/liver/kidney subtypes of AP differing only in post-translational carbohydrate modification of identical polypeptide sequences[201].

Identification of AP gene mutations in hypophosphatasia, a rare inborn error of metabolism which causes defective skeletal mineralisation, has confirmed the hypothesis that AP executes specific important functions in the process of skeletal mineralisation [199]. Analysis of serum from patients suffering from hypophosphatasia reveals normal enzyme activity in the three tissue specific isoforms of AP, but in the AP isoenzyme, a generalised reduction in activity is recorded,[202] indicating that the tissue-non-specific form of AP plays an important part in the mineralising process.

Although the precise mechanism by which AP modulates the mineralising process is unclear, a number of theories have emerged which may contribute to our understanding in this area:

AP hydrolyses nucleoside phosphate (liberated by dying cells) to increase the local concentration of inorganic phosphate (Pi)[203].

AP acts in biomineralisation by hydrolysing an inhibitor of calcification[200].

AP can act as an inorganic pyrophosphatase (PPi). At high concentrations PPi inhibits the growth of hydroxyapatite crystals[204].

AP acts as a plasma membrane transporter for Pi and a extracellular calcium binding protein that stimulates calcium phosphate precipitation into osteoid[205].

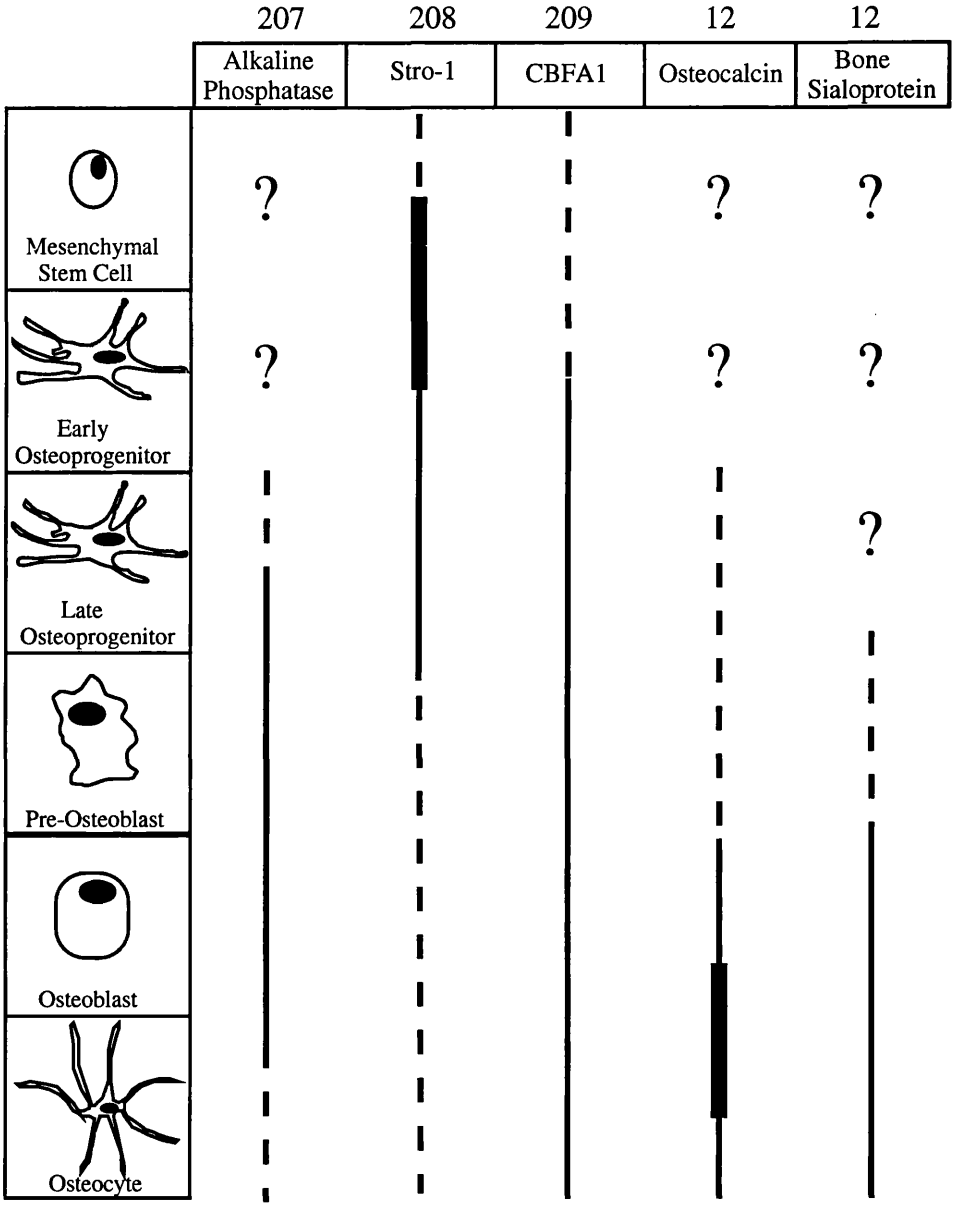
Uncertainty surrounding the precise function of AP has not impinged upon its widespread acceptance as a marker of osteogenic differentiation. It is accepted that, as the specific activity of AP in a population of cells increases there is a corresponding shift to a more differentiated state[12] and as such, is routinely used in *in vitro* experiments as a relative marker of osteoblast differentiation[14,206,207].

STRO-1 is a monoclonal antibody that recognises a trypsin resistant cell surface antigen expressed by a subset of human marrow stromal cells that includes all

assayable CFU-F. STRO-1 positive cells proliferate extensively in culture and give rise to cells of a variety of marrow stromal cell lineages[208]. These findings are consistent with the possibility that the STRO-1 population contains the primitive multipotential stem cells. The STRO-1 antigen is therefore the subject of much research and its antibody provides a useful investigative tool in this area.

CBFA1 is a recently discovered transcription factor that belongs to the core-binding factor family[209]. To date, three CBFA genes, all belonging to the Runt domain gene family, have been identified: CBFA1, CBFA2 and CBFA3[210-213]. CBFA1 knockout mice are born with a total lack of both membranous and endochondral bone, an absence of osteoblasts and extremely low expression of osteoblast associated genes[211] (alkaline phosphatase, osteopontin, osteocalcin) suggesting a key role for this factor, in early osteoblast development. This suggestion is supported by evidence that in the embryo, CBFA1 expression is located both temporally and physically, at sites of early bone development[210,211]. CBFA1 also binds specifically to OSE2, a unique osteoblast specific regulatory element found in the promoter region of all osteoblast associated genes[210]. Binding to this element induces osteoblast specific transcription and this effect further enhances the thesis that CBFA1 expression represents a specific marker of osteoblastic lineage.

Figure 1.17. Proposed Lineage Markers for Cells of the Osteoblastic Lineage.



Key: ? = Unknown; — — = Not Detected; —— = Present.

The concept of the CFU-F as a multipotential mesenchymal precursor provides a useful model in the study of *in vitro* bone cell biology. The recognition that CFU-F differentiation can be modified at the colony level *in vitro*, presents an opportunity to investigate the plasticity of differentiation between mesenchymal lineages using different factors and conditions in culture.

Because there are important differences between human and experimental animal cell culture systems, this study will focus on the use of human cells. The rationale is that human cells are most appropriate for *in vitro* work relating to human disease.

This investigation will attempt to identify factors which promote cells to the osteogenic pathways of differentiation and promote the proliferation of osteogenic cells in cultures derived from human bone marrow.

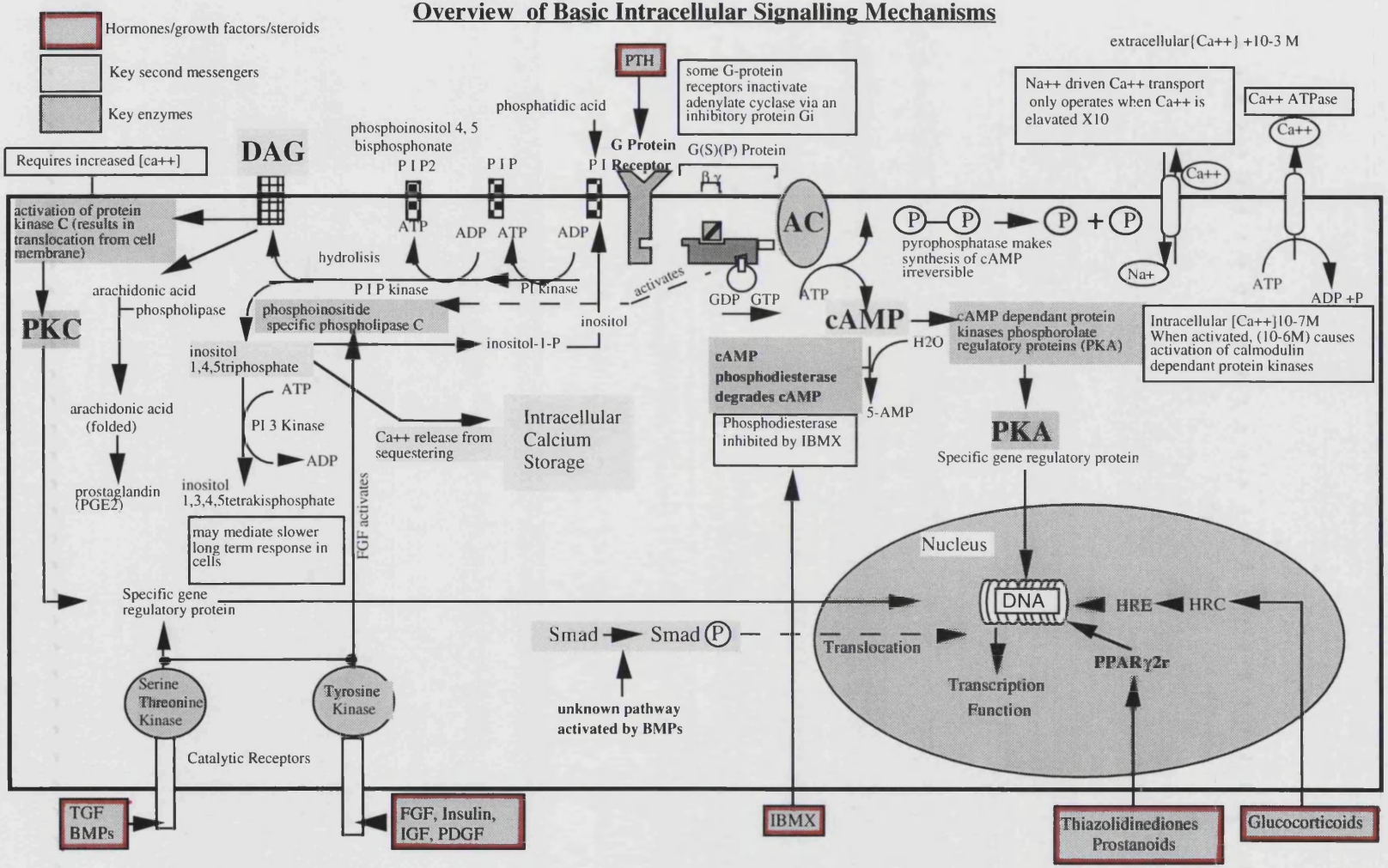


Figure 1.18: Overview of Basic Intracellular Signalling Pathways.

Chapter 2: Materials And Methods.

2.1: Materials.

2.1.1: Fixatives.

All component parts of periodate-lysine-paraformaldehyde fixative as well as paraformaldehyde were obtained from Sigma-Aldrich (Poole, UK). Methanol was supplied by Hayman PLC (Witham, UK).

2.1.2: Growth factors.

Fibroblast growth factor -2 (FGF-2) was obtained from NBS Biologicals, Insulin-like growth factor I (IGF-I) and Transforming growth factor β1 (TGFβ–1) were purchased from Sigma-Aldrich (Poole, Dorset UK).

2.1.3: Hormones.

Human Parathyroid hormone (fragment 1-34) was obtained from both Sigma-Aldrich and Eli-Lilly (Indiana, Ind. U.S.A.). Insulin was supplied by Sigma-Aldrich.

2.1.4: Immunochemicals.

The monoclonal antibody (Mab) (IgM subclass) STRO-1[208], derived from the hybridoma cell line STRO-1 was a gift from Dr Paul Simmons, Hanson Centre for Cancer Research (IMVS, Adelaide, South Australia). The STRO-1 Mab was used in the form of undiluted tissue culture supernatant. Hybridoma B4-78[214] (IgG1 subclass) which recognises the TNSAP isoenzyme of alkaline phosphatase was obtained from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA). Antibody from hybridoma B4-78 was purified from culture supernatant by protein G chromatography.

Hybridoma culture supernatant from an IgM-secreting hybridoma reactive with an intracellular antigen OBL[215] and non-immune mouse IgG1 were obtained from Sigma-Aldrich and used as negative controls.

Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM and R-phycoerythrin (RPE)-conjugated anti-mouse IgG1 were supplied by AMS Biotechnology, (Oxon. UK).

2.1.5: Kits.

Messenger Ribonucleic Acid (mRNA) was extracted from cells using the Quickprep *Micro* mRNA Purification kit supplied by Pharmacia Biotech (St Albans UK).
Protein concentration was determined using the Bio-Rad DC Protein assay obtained from Bio-Rad Laboratories (Hemel Hempstead, UK).

Intracellular cAMP levels were measured using the Biotrak cAMP enzyme immunoassay system supplied by Amersham Pharmacia (Little Chalfont, UK).

2.1.6: Molecular Biology Reagents.

Reverse transcriptase with associated buffer, RNAguard RNase inhibitor and Dithiothreitol (DTT) were all purchased from Gibco Life Sciences (Paisley, UK). reverse transcriptase random hexamer primer, (Pd(N)6), and deoxynucleotide-triphosphates (dNTPs) were supplied by Amersham Pharmacia. *Thermus aquaticus* (Taq) DNA polymerase, Taq buffer, DNA markers (ϕx Hae cut and λ Hind III cut) and magnesium chloride (MgCl) were obtained from Promega PLC (Southampton, UK). Agarose and ethidium bromide (EtBr) was supplied by Sigma-Aldrich. T4 polynucleotide kinase (PNK) and PNK buffer were supplied by New England Biolabs (Beverly, MA. USA). [γ-^{32} P] dATP and Hybond-N+ were purchased from Amersham Pharmacia. Oligonucleotide primer and probe sequences were obtained from Colin Lazarus (Dept. of Botany, University of Bristol, U.K.)

2.1.7: Staining Reagents.

Methylene Blue, Fast Red TR, alkaline buffer, napthol AS-MX phosphate and Di-methylformamide were all obtained from Sigma-Aldrich.

2.1.8: Tissue Culture Reagents.

Dulbecco's modified Eagles medium, sodium bicarbonate, Hepes buffer, L-glutamine, sodium pyruvate, trypsin-EDTA and penicillin/streptomycin were all obtained from Gibco (Paisley, UK). Fetal calf serum (FCS) was supplied by Globepharm PLC. (Esher, UK). L-ascorbate-2-phosphate (Asp)^{216} was purchased from Alpha Laboratories (Eastleigh, Hants. UK). Dexamethasone (Dx), collagenase type VII, dimethylsulfoxide (DMSO), and DNAase I were obtained from Sigma-Aldrich.

Ficoll Lymphoprep (density: 1.077g/ml), was supplied by Nycomed PLC (Birmingham, UK). Phosphate buffered saline tablets (PBS) (pH 7.4, calcium and magnesium free) were purchased from Oxoid (Bristol, UK). Tissue culture flasks and associated plastics (cell strainers, filters etc.) were obtained from Becton and Dickinson Labware (Oxford, UK) and Fahrenheit Lab Supplies (Milton Keynes).

SaOs cells were obtained from ATCC (Manassa, VA. U.S.A.)

2.1.9: Other Reagents.

Alkaline buffer, bovine serum albumin (Fraction V) (BSA), diethylpyrocarbonate (DEPC), disodium para-nitrophenol, sodium azide and sodium hydroxide (NaOH) were all supplied by Sigma-Aldrich.

Human AB serum was purchased from IBRGL Research Products (Elstree, UK), Isopropanol and hydrochloric acid (HCl) and glacial acetic acid were supplied by Hayman PLC (Witham, UK) and isoton was obtained from Coulter Electronics (Luton, UK).

2.2: Preparation of Media and Solutions.

2.2.1: Dulbecco's modified Eagle's medium + 10% (v/v) fetal calf serum (standard medium).

		Final Conc.
Dulbecco's modified Eagles medium (X10)	100.0 mls	10%
Sodium Bicarbonate (7.5%)	11.4 mls	0.085%
Hepes (1M)	20.0 mls	0.02M
Glutamine (200 mM)	10.0 mls	0.002M
Sodium Pyruvate (100 mM)	10.0 mls	0.001M
Penicillin/Streptomycin (10^4 IU/ml)	2.5 mls	25 IU/ml
Heat inactivated FCS	100.0 mls	10%

In a flow hood using aseptic technique, the above reagents were combined and made up to 1 litre with Milli Q sterile H_2O. The pH was adjusted to 7.3 using 5M NaOH and the total volume filter sterilised using a 0.22μM pore size bottle top filter. Sterility was confirmed by incubating a 5ml sample for 48 hrs at 37°C in a humidified atmosphere containing 5% CO_2.

2.2.2: Blocking Buffer.

Human AB serum	1.0 ml (10% (v/v))
Bovine serum albumin (BSA)	0.1 mg (1% (v/v))
FCS	0.5 ml (5% (v/v))

The above reagents were made up in HBSS (8.4 mls) to a total volume of 10 mls.

2.2.3: Elution Buffer (Supplied with Quickprep Micro mRNA Kit).

Tris-HCl (pH 7.5)	10 mM
EDTA	1 mM

2.2.4: Extraction Buffer (Supplied with Quickprep Micro mRNA Kit).

Buffered aqueous solution containing guanidinium thiocyanate and N-lauroyl sarcosine.

2.2.5: Freezing Solution.

FCS	50 mls (50% (v/v))
DMEM	30 mls (30% (v/v))
DMSO	20 mls (20% (v/v))

2.2.6: Glycogen Solution (Supplied with Quickprep Micro mRNA Kit).

Glycogen	10mg/ml

Made up in DEPC treated water.

2.2.7: Hank's Buffered Saline Solution (HBSS)

HBSS was obtained from Gibco Life Sciences (Paisley, UK).

2.2.8: High Salt Buffer (Supplied with Quickprep Micro mRNA Kit).

Tris-HCl (pH 7.5)	10 mM
EDTA	1mM
NaCl	0.5M

2.2.9: K Acetate Solution (Supplied with Quickprep Micro mRNA Kit).

Potassium Acetate (pH 5.0)	2.5 M

2.2.10: Low Salt Buffer (Supplied with Quickprep Micro mRNA Kit).

Tris-HCl (pH 7.5)	10 mM
EDTA	1mM
NaCl	0.1M

2.2.11: Phosphate Buffered Saline (PBS).

Five PBS tablets were dissolved in 500 ml Milli Q micronised water and the solution autoclaved.

2.2.12: Saline Sodium Citrate (SSC) (20X).

Sodium chloride	175.3 g
Sodium citrate	88.2 g

The above reagents were dissolved in 800 ml of H_2O and the pH adjusted to 7.0 with a few drops of 10N NaOH. The final volume was adjusted to 1 litre and the solution sterilised by autoclaving.

2.2.13: Serum free media.

Sodium Selenite (NaS) (2µg/ml)	1 ml (20ng/ml)
Human Apo-Transferrin (0.5mg/ml)	1 ml (5µg/ml)
BSA-Linoleic acid (5mg/ml)	100µl (5µg/ml)

DMEM was added to a total volume of 100mls and the solution was then filter sterilised using a 0.22µm pore size, bottle top filter.

2.2.14: Sodium dodecyl sulphate (SDS) (10% (w/v)).

Sodium dodecyl sulphate (electrophoresis-grade)	100 g (10% (w/v)).
H_2O	900 ml (90% (v/v))

The reagents were heated to 68°C to assist dissolution, pH was adjusted to 7.2 using a few drops of concentrated HCl . The final volume was then adjusted to 1 litre.

2.2.15: Tris-Acetate (TAE) (10X).

Tris base	48.44 g (0.4 M)
Sodium acetate	4.10 g (0.05 M)
EDTA (0.5 M, pH 8.0)	3.72 g (0.05 M)

The above were dissolved in dH_2O to a final volume of 1 litre and the pH adjusted to 8.1 with glacial acetic acid.

2.2.16: Tris-Borate Buffer (TBE) (5X).

Tris base	54.0 g (0.445 M)
Boric acid	27.5 g (0.445 M)
EDTA (0.5 M, pH 8.0)	20.0 ml (0.01 M)

The reagents were dissolved in dH_2O and made up to a final volume of 1 litre.

2.2.17: Wash Buffer (Flow Cytometry).

HBSS	950 ml (95% (v/v))
FCS	50 ml (5% (v/v))

2.2.18: Human Platelet Poor Plasma (PPP).

Human platelet poor plasma was always prepared fresh for immediate use according to the following protocol: Donor lying down and rested. Using a syringe and needle, not a vacutainer, 50 ml of whole blood is taken and gently transferred to a Falcon tube containing trisodium citrate as anticoagulant (0.105 M at 1:8 ratio in sample). The sample should then be processed as soon as possible.

Centrifuge at 1000g for 30 mins at 0°C. Remove the plasma, add to fresh tube then repeat centrifuge process. Remove remaining plasma, use immediately.

2.3: Methods.

2.3.1: Tissue Culture.

General conditions.

Unless otherwise described, all procedures described were carried out using aseptic technique in a Microflow category II flow hood. All cell types were cultured at 37°C in humidified atmosphere comprising 95% air, 5% CO_2.

Preparation of bone marrow mononuclear cells (BMSC)

Bone marrow was obtained from segments of rib removed during routine thoracic surgery at Frenchay Hospital, Bristol. Patients used for this study had no history of metabolic bone disease or treatment with drugs known to affect bone metabolism. Samples were obtained in accordance with procedures approved by the local ethics committee.

In a flow hood, using aseptic technique, the ribs were cleared of all extraneous muscular and connective tissue. The ribs were then divided and the marrow cavity and flushed using PBS. The resulting cell suspension was centrifuged at 500g for 10 mins. BMNC were isolated from the cell pellet following resuspension and density gradient centrifugation on Ficoll Lymphoprep (600g, 30 mins). The BMNC layer was extracted from the Lymphoprep/PBS interface, washed twice in PBS by centrifugation (500g, 10 mins) and a suspension of single cells obtained by filtration through a 45 µm pore size, cell strainer. Cell numbers were determined using a Coulter Counter electronic cell counter (Coulter Electronics PLC, Luton UK).

Maintenance of Cells.

BMNC and SaOs-2 cells were initially seeded in culture flasks at a density of 2 X $10^4/cm^2$ in standard medium supplemented with 100µm ascorbate-2-phosphate and test factors. After 7 days in culture the non-adherent cells were removed and the medium replenished twice weekly thereafter.

Passaging and Harvesting of BMNC.

When passaging or harvesting cells, the medium was removed and the cell layer rinsed twice with PBS. The cells were then incubated with DMEM (10mls/T75) containing 30 IU/ml collagenase type VII and 2mM additional Ca^{++} for 1 hour at 37°C. Following the removal of the collagenase solution the cells were washed twice with PBS and then incubated with 0.02% trypsin-EDTA (5mls/T75) for 5 mins. After checking that cell detachment had occurred, the trypsin reaction was terminated by

the addition of 1% (v/v) FCS. A single suspension was achieved by gentle pipetting and straining using a Falcon 70μm cell strainer. The cells were then harvested, washed in PBS by centrifugation (5 min, 500g) and counted.

Passaging Cell Lines.

When passaging SaOs cell lines the collagenase step was omitted.

Freezing of cells.

Cells were harvested, washed and counted, then resuspended in 50% (v/v) FCS / 50% (v/v) DMEM to a concentration of 1 X 10^6 /ml . 500μl aliquots of the suspensions were placed in freezing vials where freezing solution was added in a dropwise fashion to a total volume of 1 ml. Vials were then cooled slowly overnight to -70°C before transfer to storage in liquid nitrogen .

To set up cells from frozen, a vial was removed from the liquid nitrogen and rapidly defrosted by clasping in the hand. The vial was then washed in 70% (v/v) ethanol before the cells were removed and washed twice in DMEM by centrifugation (500g, 10 mins). The cells were then plated into culture flasks containing standard medium and re-established in culture and expanded. These cells were then harvested for use in experiments.

2.3.2: Bioassays.

Alkaline Phosphatase (AP) Activity Assay.

Cellular alkaline phosphatase activity was measured colorimetrically by monitoring the release of para-nitrophenol (p-nitrophenol) from disodium p-nitrophenol phosphate at 37°C, pH 10.5. The reaction produces a coloured product which can be measured spectrophotometrically at 410 nm.

Cells were cultured for various times under test conditions in 96 well plates. At the end of the treatment period, the medium was removed and the cells washed twice in PBS. Alkaline buffer (100μl/well) was added and incubated at 37°C for 30 min. Phosphatase substrate containing 4mg/ml disodium p-nitrophenol phosphate was prepared in ddH_2O containing 2μM MgCl and added to the wells (100μl/well). A set of standards using known concentrations of p-nitrophenol in alkaline buffer in the range 1-200nM, was set up on the plate in parallel. Blanks were prepared by adding 100μl of alkaline buffer to 100μl of substrate solution.

A further incubation at 37°C continued until the colour intensities of the samples were comparable with the standards. The reaction was then stopped using 50μl, 1M

NaOH/well and the incubation time recorded. The absorbance was read at 410nm on a Dynatech MR5000 plate reader and enzyme activity, expressed as nMol/p-nitrophenol/min, determined from the standard curve (fig. 2.1).

cAMP Assay.

Intracellular cyclic adenosine monophosphate (cAMP) was assessed using the Biotrak cAMP enzymeimmunoassay (RPN 225) supplied by Amersham Life Sciences.
The basis of this assay is the competition between unlabelled cAMP and a fixed quantity of peroxidase-labelled cAMP. The labelled and unlabelled cAMP compete for a limited number of binding sites on a cAMP antibody. With fixed amounts of antibody and peroxidase-labelled cAMP the amount of peroxidase-labelled ligand bound by the antibody will be inversely proportional to the concentration of added unlabelled ligand. The peroxidase ligand that is bound to the antibody is immobilised on to polystyrene microtitre plates precoated with second antibody (fig. 2.2a), thus the unbound ligand can be removed by a simple washing procedure

The amount of peroxidase labelled cAMP bound to the antibody is determined by the addition of a tetramethylbenzidine (TMB)/hydrogen peroxide single pot substrate The reaction is stopped by the addition of an acid solution, the resultant colour read at 450 nm in a microtitre plate spectrophotometer.
Concentration of cAMP in f/Mol was determined from a standard curve generated as part of the assay (fig. 2.2b). cAMP concentrations were then normalised to fMol/mg cell protein, using the Bio Rad DC protein assay.

Preparation of Cells for cAMP Assay.

Cells to be assayed were plated at a density of 1.25 x 10^5 cells/well in a 24 well plate and left overnight to adhere in standard culture conditions. The following day at T -15 mins the media was removed and replaced with serum free medium (SFM) containing 500μM iso-butyl-methyl-xanthine (IBMX). At T = 0 the SFM/IBMX media was removed and replaced with SFM/IBMX containing the PTH treatments. The reaction was terminated at T +15 mins by the addition of absolute ethanol to 66% (v/v) at a temperature of -20°C. The ethanol extract was removed and added to a 5 ml microfuge tube, then spun at 500g for 10 mins at 4°C. The ethanol supernatant was then evaporated on a Jouan RC. 10.22 rotary evaporator at 50°C for 6 hours using a pulsed vacuum. The resulting extract was dissolved in assay buffer and assayed according to kit instructions.

Figure 2.1: A Typical Standard Curve for the AP Activity Assay

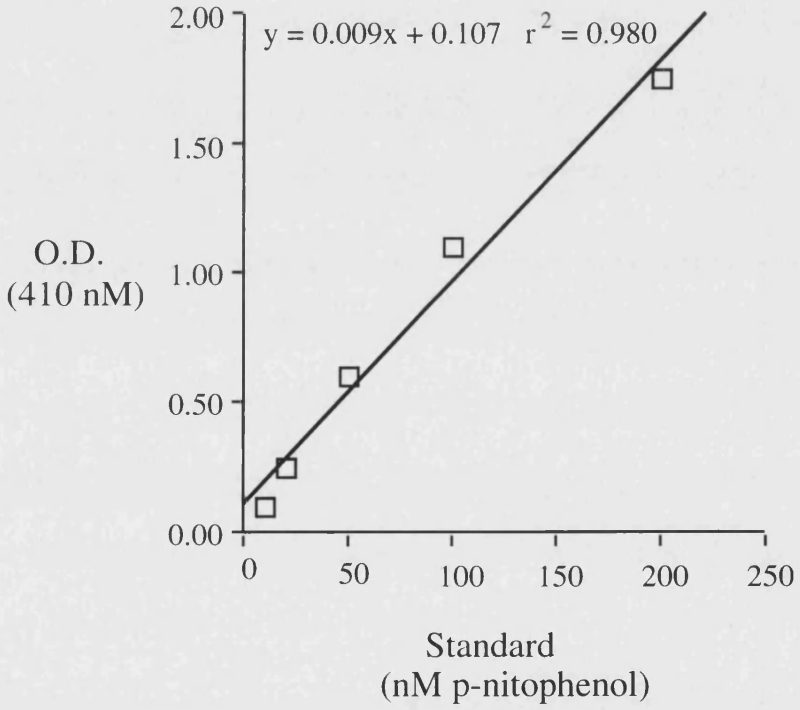


Figure 2.2a: Schematic Representation of Antibody Binding in the Biotrak cAMP Assay

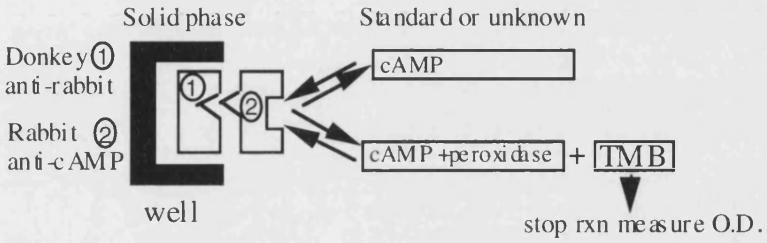
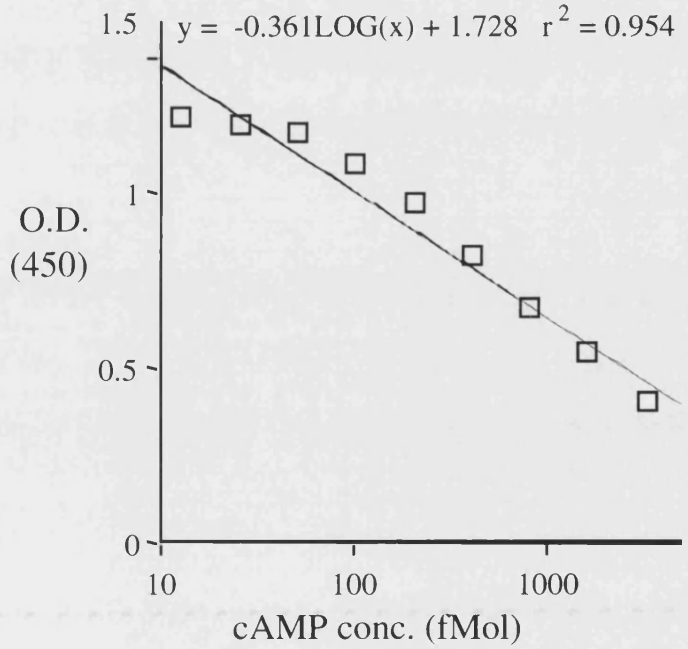


Figure 2. 2b: Typical Standard Curve for the cAMP Assay



Bio-Rad DC Protein Assay

Bio-Rad DC Protein Assay is a colorimetric assay used to determine protein concentration following detergent solubilisation. The reaction is similar to the well documented Lowry Assay[217].

The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. As with the Lowry assay, there are two steps which lead to colour development: The reaction between protein and copper in an alkaline medium and the subsequent reduction of Folin reagent by the copper treated protein[218]. Colour development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cysteine and histidine. Proteins effect a reduction of the folin reagent by a loss of 1, 2 or 3 oxygen atoms, thereby producing one or more several possible reduced species which have a characteristic blue colour with maximum absorbance at 750nM and minimum absorbance at 405nm.

Samples were set up in triplicate and cultured for various times under test conditions in 96 well plates. At the end of the treatment period, the medium was removed and the cells washed twice in PBS. To each well was then added 25 µl of alkaline tartrate solution and 200 µl of a dilute folin reagent (both reagents supplied with kit). After 15 minutes at room temperature the absorbance was read at 750nm and protein concentration determined from a standard curve.

The standard curve was set up using triplicate BSA protein concentrations in the range 0.25 mg/ml - 4 mg/ml. Figure 2.3 shows a typical standard curve.

MTT Rapid Colorimetric Assay

Cell proliferation was measured in 96 well plates using MTT (3-(4,5 Dimethylthiazol-2y)-2,5-diphenyl tetrazolium bromide), a pale yellow substrate which produces a dark blue formazan product when incubated with live cells[19]. The amount of formazan product produced is directly proportional to the number of live cells. The formazan product is only partly soluble in the medium and must be dissolved to produce a homogenous solution suitable for measuring its optical density.

Samples were set up in triplicate and cultured for various times under test conditions in 96 well plates. At the end of the treatment period, the medium was removed and the cells washed twice in PBS. 20 µl of MTT (5mg/ml) was then added to each well and incubated for 4 hours at 37°C. The reaction was then stopped using 100µl, 10% SDS.

Figure 2.3: Typical Standard Curve for the Biorad Protein Assay

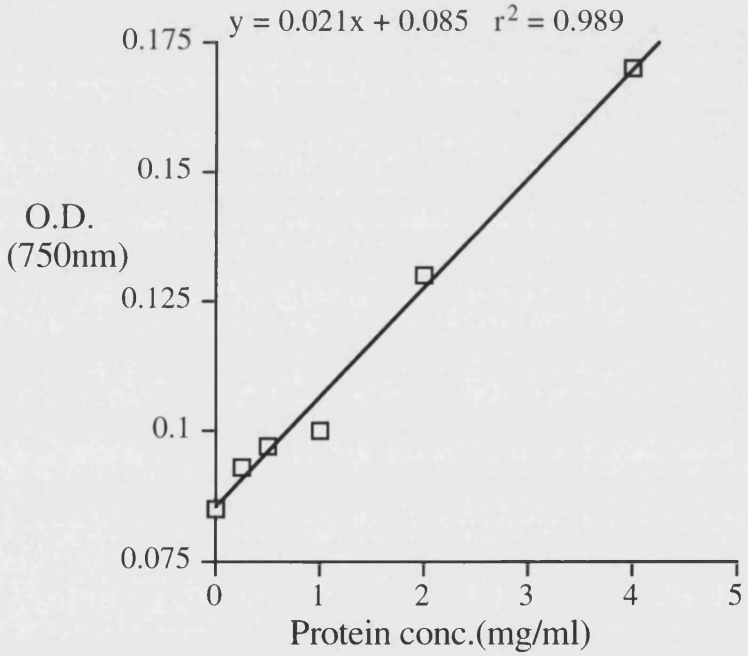
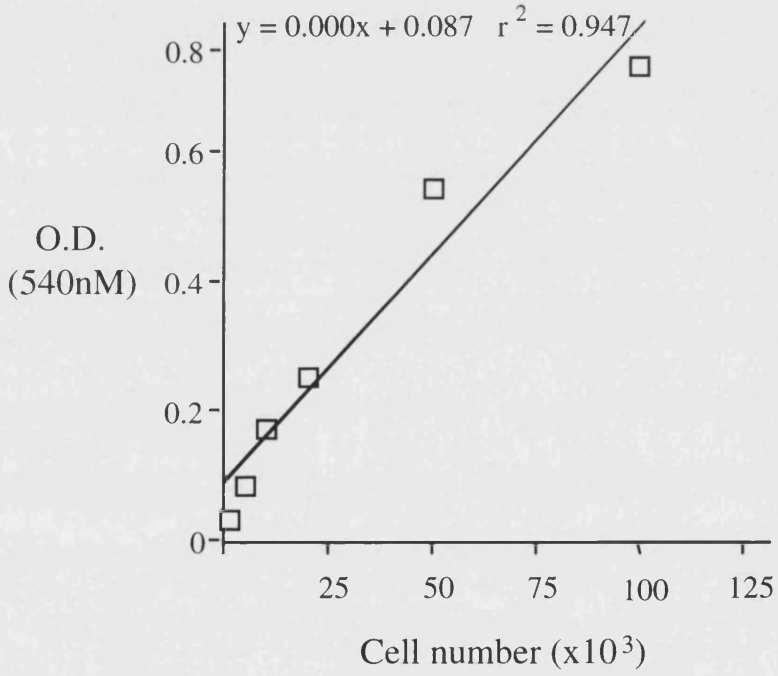


Figure 2.4: A Typical Standard Curve for the MTT Proliferation Assay.



in 0.01M HCl. Dissolution of insoluble MTT component was achieved by overnight incubation in the SDS solution. The optical density was then read on a Dynatec plate reader using a reference wavelength of 630nm and a test wavelength of 570nm.

Optical density is directly proportional to cell number thus allowing direct comparisons of proliferation to be made.

2.3.3: Flow Cytometry.

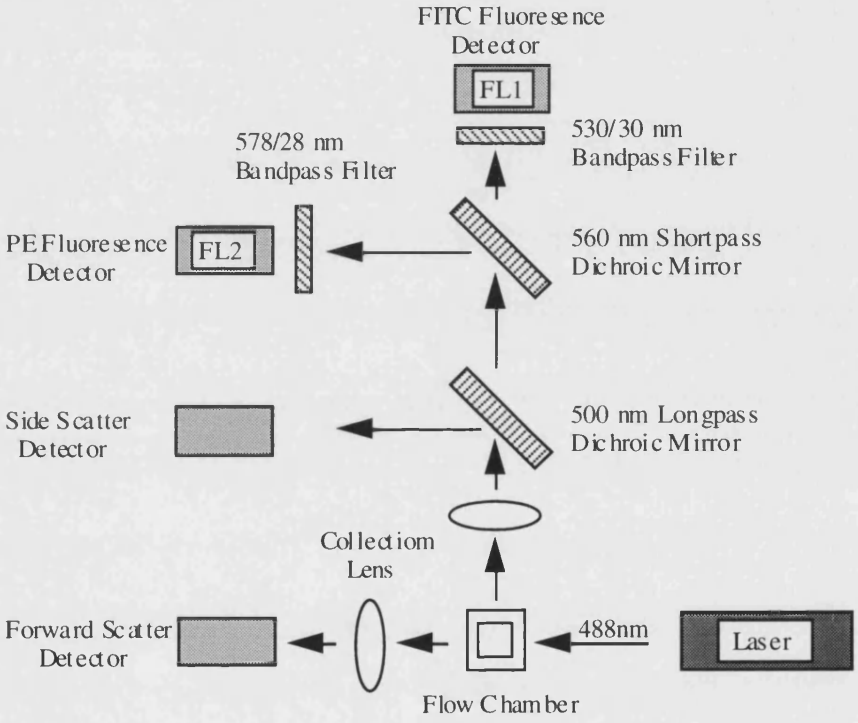
Flow cytometry analysis was carried out on a Becton Dickson FACStar Plus flow cytometer, equipped with a 488nm, 100mW argon ion laser and Consort computer. Flow cytometry allows the rapid measurement of multiple cellular parameters based on light scatter and fluorescence in a stream of cells passing through a laser illuminated sensing point[220] (fig. 2.5). Laser illumination of specific wavelength excites fluorescent dyes (e.g.. fluorescein isothiocyanate (FITC) and R-phycoerythrin (PE) conjugated to the cell and the resulting scattered and fluorescent light is collected by photodetectors and converted into electronic signals. Optical filtration permits the separation of scattered and fluorescent light allowing specific, independent but correlated measurements to be made.

Measurements of forward scattered light (FSC) relate to cell size and side scattered light (SSC) values relate to cell granularity. Conjugation of fluorescent dyes to ligands and antibodies enables the density and distribution of cell surface and cytoplasmic determinants to be studied. Data was collected as either dot plots or frequency histograms (fig. 2.6). Dot plots consisted of SSC versus FSC graphs allowing the identification of discrete cell populations, or FL1 (FITC channel) versus FL2 (PE channel). Histograms generally depicted fluorescence intensity..

Preparation of Cells for Flow Cytometry.

Cells were cultured for differing times under various test conditions in T75 culture flasks. At the end of the treatment period the medium was removed and the cells washed twice in PBS, then harvested as previously described.

Figure 2.5: Typical Configuration for the Measurement of Scattered light, FITC and PE Immunofluorescence [220].

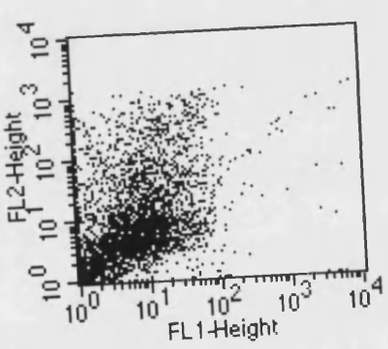


FL1 = Fluorescence Channel 1

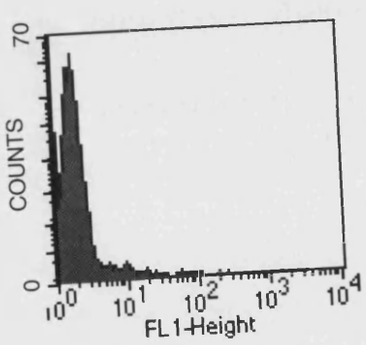
FL2 = Fluorescence Channel 2

Figure 2.6: Typical Dot Plot and Histogram used in Flow Cytometry Analysis.

(a) Typical dot plot.



(b) Typical histogram



Immunolabelling of Cells for the Detection of STRO-1 and AP Expression.

Cells were immunolabelled with monoclonal antibodies recognising either the STRO-1 antigen (STRO-1), the AP antigen (B4-78) or both. After harvesting, the cells were washed once in wash buffer and pelleted by centrifugation (400g, 5 mins). The cells were then incubated on ice for 30 mins in blocking buffer to reduce non-specific antibody binding. The cells were then aliquoted into 5 ml FACS tubes at 1 x 10^5 cells/tube and washed by centrifugation (400g, 5 mins) with 4 mls of wash buffer. The buffer was aspirated and the cells incubated for 1 hour on ice with saturating concentrations of mouse IgG1 in STRO-1 Mab (20μg/ml) and/or B4-78 Mab in OBL (20μg/ml). Mouse IgG1 in OBL (20 μg/ml) was used in the negative controls.

The cells were then washed with 4 mls of ice cold wash buffer by centrifugation before the addition of 200 μl of a combination of optimally diluted FITC conjugated anti-mouse IgM μ–chain specific (1/50 dilution in wash buffer) and PE-conjugated goat anti-mouse IgG2a specific (1/200 dilution in wash buffer) immunoglobulins. The cells were incubated for 45 mins on ice and then washed by centrifugation (500g, 5 mins) before being analysed by flow cytometry. For dual labelled samples, compensation parameters were set using cells stained solely for STRO-1 or AP expression.

In all flow cytometry experiments, data acquisition and analysis were performed using the LYSYS II ™ and CELL QUEST software packages.

2.3.4: Preparation and Analysis of mRNA and DNA.

mRNA Extraction.

mRNA extraction was achieved using a Quickprep *Micro* mRNA Purification Kit (Pharmacia, St Albans U.K.). Briefly, the cells were harvested as previously described and extracted in a buffered solution containing a high concentration of guanidinium thiocyanate (GTC), this ensured the rapid inactivation of endogenous ribonucleic acid nucleases (RNases). The extract was then diluted three-fold with Elution Buffer, reducing the GTC concentration to a carefully selected level allowing the efficient hydrogen-bonding between poly(A) tracts on the mRNA molecules and oligo (dT) attached to cellulose. The extract was then clarified by a short centrifugation and the supernatant transferred to a microfuge tube containing oligo (dT) cellulose.

After several minutes, during which the poly $(A)^+$ RNA binds to the oligo (dT) cellulose, the tube was centrifuged at 16,000g for 10 seconds, and the supernatant

removed. Subsequent washes (3 X 0.5 ml) in both high and low salt buffers were followed by the elution of mRNA from the cellulose using pre-warmed elution buffer (0.2ml X 2). mRNA was then concentrated by precipitation with the addition of 10µl of glycogen solution, 40µl of K acetate solution and 1 ml of 95% (v/v) ethanol (-20°C) to 400µl of sample. The sample was then placed at -80°C overnight. Precipitated mRNA was then collected by centrifugation at 16,000g for 5 mins at 4°C. After aspirating the supernatant, the mRNA pellet was re-dissolved in DEPC-treated water.

The concentration of mRNA following the precipitation step was determined by spectrophotometry. 10µl aliquots were diluted to 250 µl in DEPC treated water and the absorbances read at 260 and 280nm in a LKB Ultraspec II spectrophotometer and the A_{260}/A_{280} ratios calculated. A ratio of 2.0 is expected, lower values indicate impurities or contamination by protein.

Reverse Transcription of mRNA.

mRNA was reverse transcribed to form cDNA prior to amplification in the polymerase chain reaction (PCR). 100 ng of mRNA was added to an eppendorf tube containing 5.0 µl (5X) Reverse transcriptase buffer, 100 IU reverse transcriptase, 10 mM dNTPS, 10mM DTTs, 10mM Pd (N) 6 and 35 IU/µl RNAguard RNase inhibitor. This volume was made up to 25 µl with DEPC treated water and overlayed with mineral oil to prevent evaporation. The tube was then placed in a thermal cycler (Stratagene 96 Robocycler) where the reaction was held at 37°C for 90 mins. The reaction was then terminated by heating to 94°C for 20 mins when the samples were then used directly in the PCR reaction or, stored at -20°C for future use.

Amplification of cDNA [221].

2µl of cDNA was added to an eppendorf containing 2.5 µl (10X) Taq polymerase buffer, 0.1µl Taq polymerase[222], 2.5 µm MgCl and 0.5mM dNTPs. Two oligonucleotide primers specific for the particular DNA sequence of interest were also added to a concentration of 1µM each. The volume was made up to 25 µl and overlaid with mineral oil. Amplification was carried out in a thermal cycler (Stratagene 96 Robocycler) where the temperatures, times, and cycle numbers of the denaturing, annealing and extension steps differed according to the primer used.

Agarose Gel Electrophoresis.

Horizontal submarine gel electrophoresis was used for the analysis of DNA [223].

2% (w/v) Flowgen Agarose (routine electrophoresis grade) was added to 1X TAE buffer and heated until melting point. The solution was allowed to cool to

approximately 60°C when ethidium bromide was added to a concentration of 0.5µg/ml. Ethidium bromide contains a planar group which intercalates between the stacked bases of DNA causing the fluorescent intensity of the ethidium to increase, allowing the DNA product to be visualised under U.V. light.

The cooling agarose was then poured into a perspex mould and a comb placed in the appropriate slot. The agarose was allowed to set at room temperature when the comb was removed and the gel placed into an appropriate running tank with the sample wells towards the cathode. The gel was then covered in 1X TAE, the running buffer.

DNA samples were loaded in DNA loading dye (15% (w/v) ficoll, 0.25% (w/v) xylene cyanol, 0.25% (w/v) bromophenol blue) and electrophoresed at 90V until the dye front had travelled at least halfway across the gel.

After electrophoresis, the Southern Blot technique[224] was used to detect the presence of specific DNA sequences in the gel. In this process, DNA in the gel is denatured and transferred by capillary action from the gel to a nitocellulose membrane where it then binds (fig. 2.7).

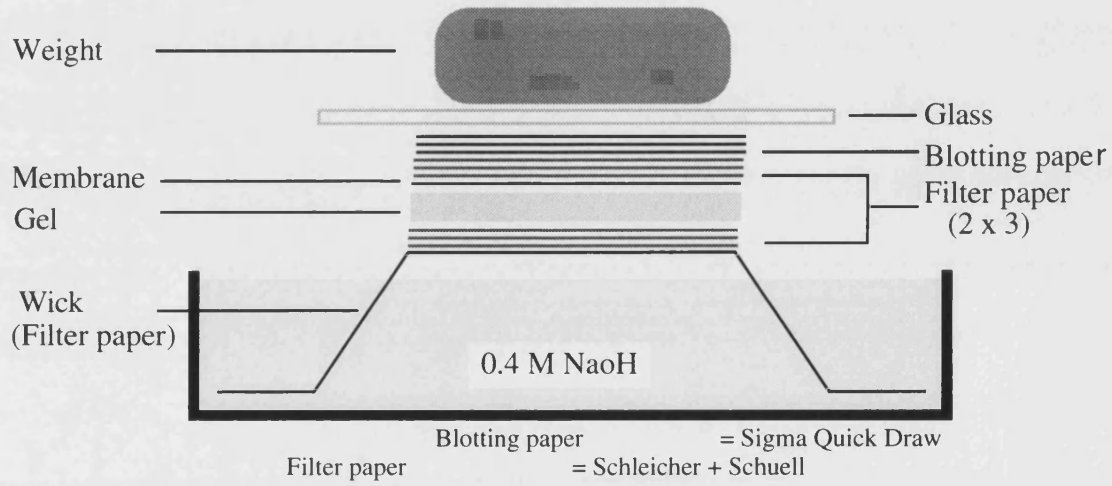
The gel was removed from the tank and placed with the DNA uppermost against the Hybond-N+ nitrocellulose membrane. The Southern Blot was constructed as shown in figure 2.8 and care was taken to exclude air bubbles between the layers. The blot was left overnight before the apparatus was carefully dismantled and the membrane washed twice in SSC.

Probing the Membrane.

After washing, the membrane was incubated with pre-hybridisation buffer (20 ml (6X) SSC containing 5 mg non-fat dried milk (0.025% (w/v)) at 65°C for 2 hours. The membrane was then rinsed with 6 X SSC containing 0.5% SDS.

A radioactive probe was constructed by combining 5 pMol oligonucleotide complementary to the DNA sequence of interest, 1 µl 10X PNK buffer, 1 µl/ml T4 PNK, 1 µl γ^{32}P ATP and 6 µl dH20. Incorporation was achieved by incubation at 37°C for 30 mins then 65°C for 20 mins. The labelled probe was then separated from the unincorporated using a Nick column.

Figure 2.7: Southern Blotting Apparatus.



The membrane was then incubated overnight at 65°C in 5 mls of hybridisation buffer containing 10 µl of probe. The following day the probe was removed and the membrane was washed twice in 2 X SSC containing 0.05% SDS, then twice in 0.1 SSC containing 0.05% SDS and finally, once in 0.1 X SSC. The membrane was then sealed in plastic and exposed to Fuji medical X-ray film in a Cronex Quanta Fast Detail Cassette autoradiography cartridge (DuPont).

2.3.5: Fixing and Staining of Colonies and Cells.

Fixation of colonies

Following the aspiration of medium, colonies were washed twice in PBS before fixation for 5 mins at room temperature in absolute methanol. The methanol was aspirated and the colonies allowed to air dry before the addition of the relevant stain.

Staining AP Positive (AP+) Colonies.

Using glassware, Napthol AS-MX phosphate was added to dimethyl formamide to achieve a concentration of 10mg/ml before dilution (1: 50) in 0.1M Tris buffer. Fast Red salt (1mg/ml) was dissolved in the solution and filtered before addition to the colony plates. After 10 mins at room temperature the stain was washed off using liberal amounts of dH_2O. Colonies expressing AP were stained a red/pink colour.

Staining of Colonies.

Colonies were visualised in culture flasks after staining with 1% (w/v) methylene blue in 10mM tris-borate buffer. After 30 mins at room temperature, the stain was washed off using liberal amounts of dH_2O leaving all colonies stained a dark blue colour.

Light microscopy.

Colonies and cells were visualised using standard light microscopy techniques on a Leitz Fluorovert FS inverted microscope fitted with a 35mm camera. Photographs were taken on 35mm 100ASA tungsten transparency film and processed commercially.

Image analysis

Image analysis of colony areas was carried out manually on a G3 Macintosh computer using the image analysis software Image v. 1.6.1. Image is distributed freely and is downloadable from http://rsb.info.nih.gov/nih-image/

Chapter 3: Testing Of Serum Supplements.

3.1: Introduction.

The ability to grow mammalian cells in culture has been vital for increasing our understanding of cell biology and biochemistry. As the growth requirements of normal cells in culture are not well characterised, there is almost a universal requirement to supplement media preparations with serum proteins[225]. Serum is thought to be effective in supporting cultured cells because it contains a large number of different growth promoting factors in a physiologically balanced blend[26]. The importance of serum supplements in mammalian culture systems has been illustrated by experiments showing that when cells are exposed to serum free medium for a short time, they leave the cell cycle[226]. Because serum is poorly characterised, the following experiments were undertaken to establish a suitable serum for use in this study. Both fetal calf sera (FCS) and, because of the intention to use primarily human cells in the study, platelet poor human plasma (PPP) were considered.

3.2: Methods.

Samples of 4 different fetal calf sera were obtained from relevant suppliers and compared, using markers of proliferation and osteogenic differentiation, to a serum currently in use and known to support osteogenic cells in culture. The sera tested were:

(i) Globepharm, batch number 2704.
(ii) TCS, batch number 01005.
(iii) ICN, batch number 1004011.
(iv) Imperial, batch number 42342.

The following parameters were compared:

(i) Cell numbers.
(ii) Cell proliferation.
(iii) Colony forming efficiency (Total number of colonies and AP expressing colonies).
(iv) AP activity, a marker of osteogenic differentiation.

Bone marrow cells were obtained as described previously, from segments of rib removed during routine thoracic surgery from a male patient aged 59 at Frenchay Hospital, Bristol. The cells were seeded in standard media formulation plus 100μM L-ascorbate-2-phosphate with, and without, dexamethasone (10^{-8}M unless otherwise stated). The media preparation was supplemented with the appropriate test sera (10% v/v).

Cells were plated at a density of 2 X $10^5/cm^2$ in T75 culture flasks (x5 / serum) and cultured for 21 days to assess serum effects on cell numbers. A similar plating density was used in 6 well plates (6 wells / serum) where cells were cultured for 21 days to estimate the effects on colony forming efficiency (CFE). Cells were plated in 96 well plates (1 X 10^5/well, 5 wells / serum) for both the cell proliferation and the AP activity assays. Estimations of both these parameters were measured at 7, 14 and 21 days.

Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. After 7 days in culture, the non-adherent cells were removed then replenished with appropriate medium twice weekly thereafter. At the appropriate time point, the cells were harvested and parameters assessed.

Cell numbers were counted on a Coulter counter electronic cell counter. Colonies were fixed and stained to evaluate the total number and AP expressing, proliferation was measured using the MTT assay and AP activity was assessed by monitoring the release of p-nitrophenol from di-sodium phosphate. All these processes were carried out as described in Chapter 2.

All experiments were repeated at least once and data from a single representative experiment is shown. The distribution of the data was confirmed as normal using the Shapiro Wilk W test, where data was not normally distributed, a log transformation was used to normalise the distribution prior to test. Differences in the data were assessed using the one way Analysis of Variance followed by the Tukey-Kramer test of Honest Significant Difference ($p < 0.05$).

3.3: Results (Fetal Calf Sera).

3.3.1: Cell Numbers .

Cultures supplemented with the standard serum yielded the greatest number of cells after 21 days in culture, rank order: Standard > serum 2 > serum 1 > serum 3 > serum 4 (fig. 3.1). The standard serum, serum 1, serum 2 and serum 3 did not differ significantly from each other whilst serum 4 yielded significantly lower numbers than the standard, 1 and 2 ($p < 0.05$).

When the sera were further supplemented with dexamethasone (Dx), serum 2 yielded the greatest number of cells, rank order: Serum 2 > 1 >standard > 4 > 3. These differences were not significant.

3.3.2: Cell Proliferation .

Cell proliferation was assessed from O.D.s.(fig. 3.2) measured at 540 nm after 7 days, 14 days and 21 days in culture. Interpolating from the standard curve (fig. 3.3) cell numbers were estimated and no significant difference in the rate of proliferation was noted between any of the tested sera at any time point (fig. 3.4a). When further supplemented with Dx (fig. 3.4b), no significant inter-sera differences in proliferation were evident at any time point. After 21 days in culture the rank order of sera proliferation was: Serum 2 > standard > 3 > 1 > 4. In Dx supplemented sera, the rank order was: Standard > 3 > 1 > 4 > 2.

3.3.3: Colony Numbers.

Total colony numbers and AP expressing colonies were assessed after 21 days in culture and the rank order of sera in the total colony category was: Standard> 1 > 4 > 2 >3 (fig. 3.5a). Cultures containing the standard serum and serum 1 had significantly ($p < 0.05$) more colonies than cultures containing sera 2, 3, and 4. The addition of Dx (fig. 3.5a) to the cultures caused an overall slight increase in colony numbers for all groups except serum 4 where the number of colonies increased significantly.

3.3.4: AP+ Colony Numbers.

Cultures containing colonies expressing AP were ranked: serum 1 > standard > 4 > 2 > 3 (fig. 3.5b). Serum 1 and the standard contained significantly more AP expressing colonies than the others. In Dx supplemented sera (fig. 3.5b), the rank order was: Standard > Serum 1 > 4 > 2 > 3. Again the standard and serum 1 had a significantly higher number of AP expressing colonies.

Expressing the number of AP colonies as a percentage of the total colony number (fig. 3.6) ranked: serum 1 > standard > 3 > 2 > 4. In Dx supplemented cultures, the standard = serum 1 > 4 > 2 > 3.

3.3.5: AP Activity.

Cultures were measured for AP activity at 7, 14 and 21 days (fig.3.7 a,b,c). No significant inter-serum differences in AP activity were seen at any time point whether +/- Dx. After 21 days in culture the rank order was: Serum 4 > 1 > 2 > 3 > standard. The Dx supplemented cultures were ranked: Standard > 1 > 3 > 4 > 2.

Figure 3.1: The Effect of Different Sera on BMSC Numbers.

Unless otherwise stated: Bars represent S.E. of mean; n = 4

* $p < 0.05$ compared to relevant control

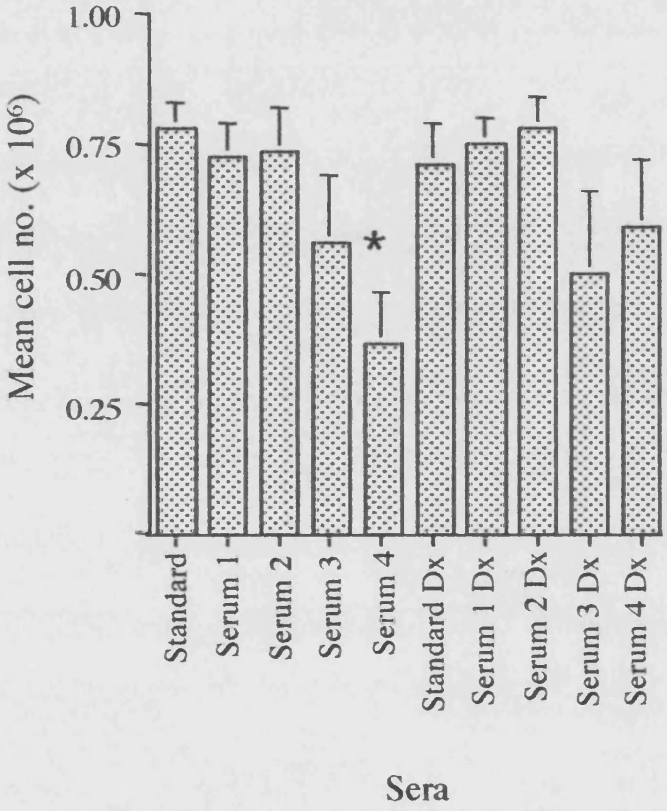
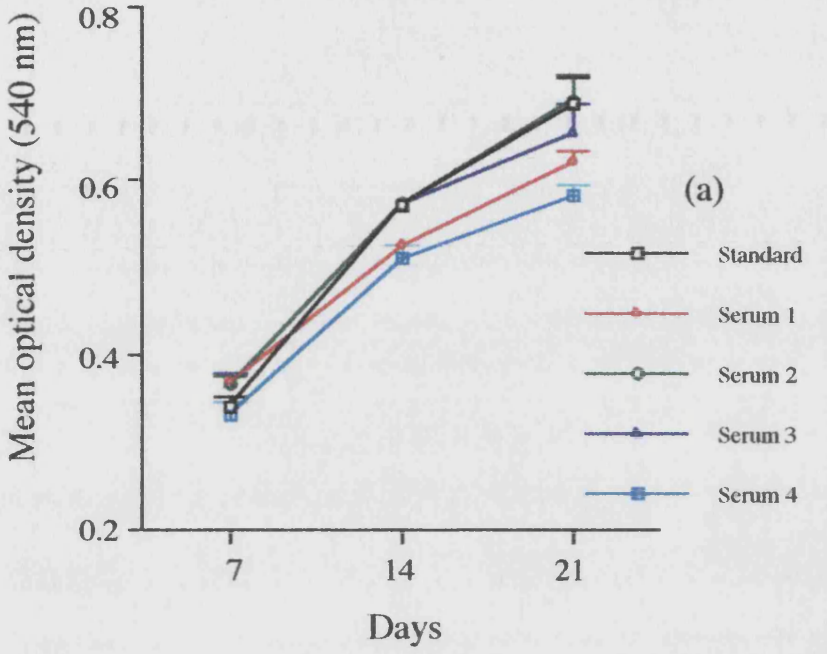


Figure 3.2: The Effect of Different Sera on the Proliferation of BMSC after 7, 14 and 21 days in Culture @ O.D. 540 nM.



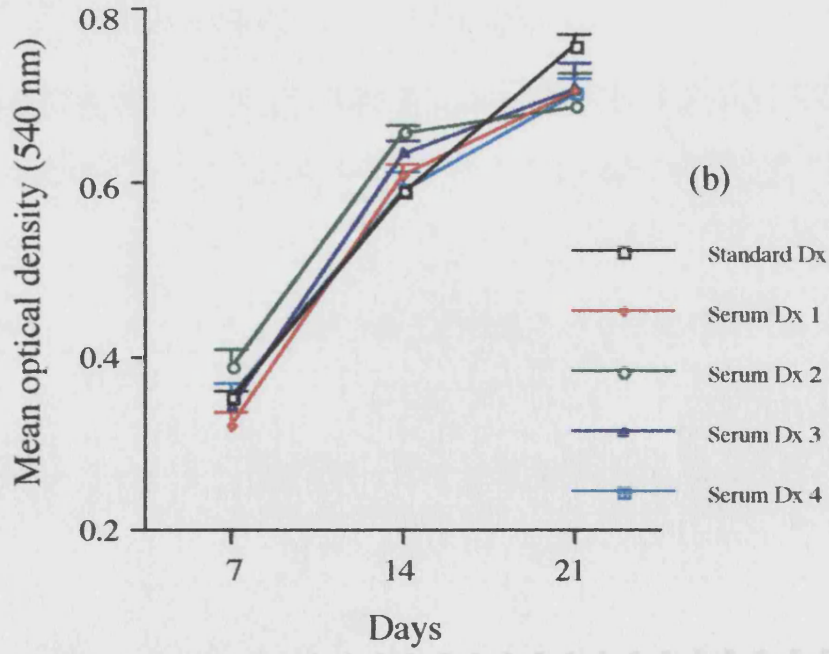


Figure 3.3: Standard Curve for the MTT Assay of Cell Proliferation.

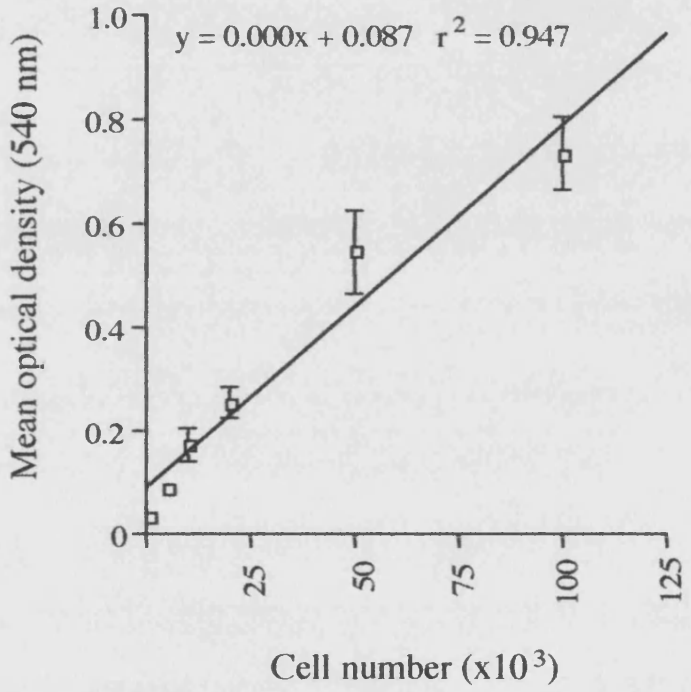
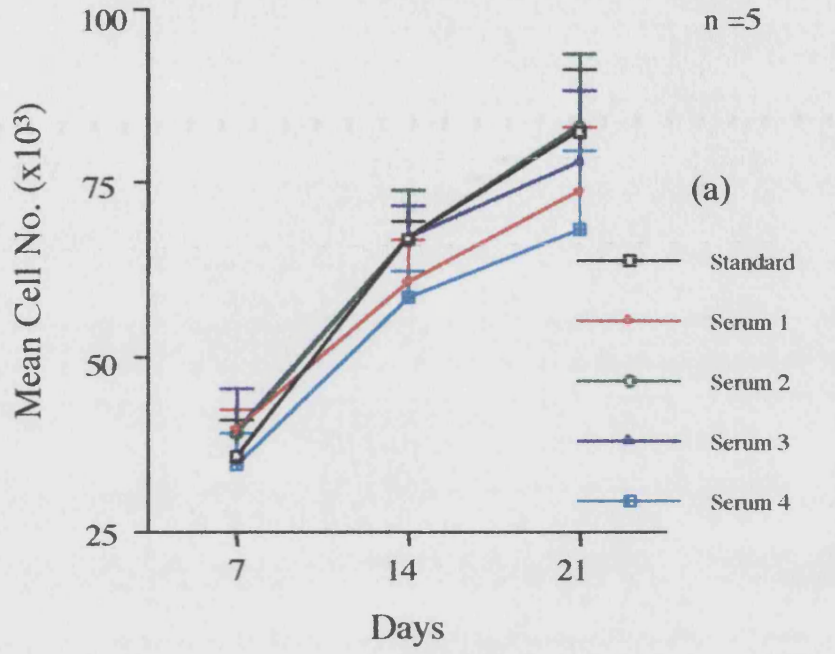


Figure 3.4: The Effect of Different Sera on the Proliferation of BMSC after 7, 14 and 21 days in Culture: Interpolated Cell Numbers.



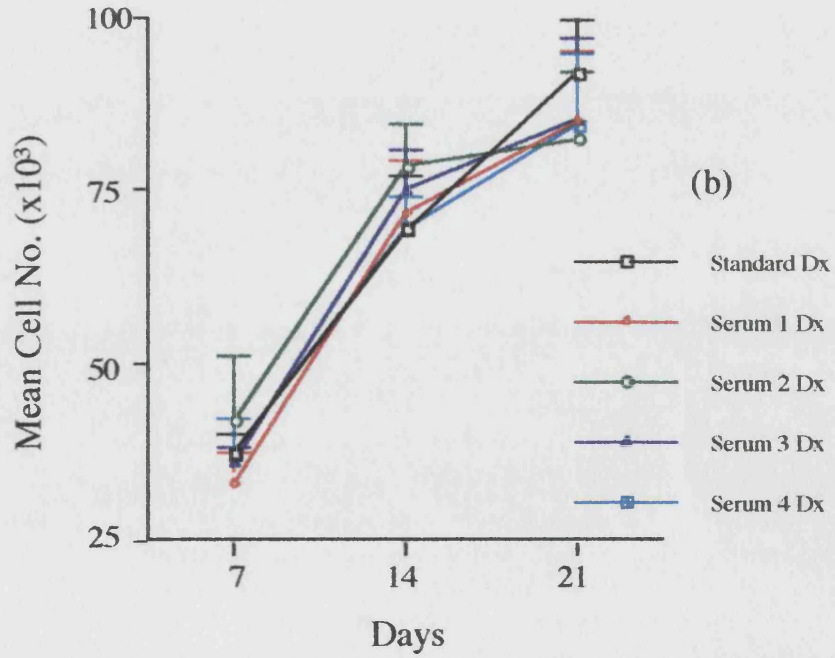
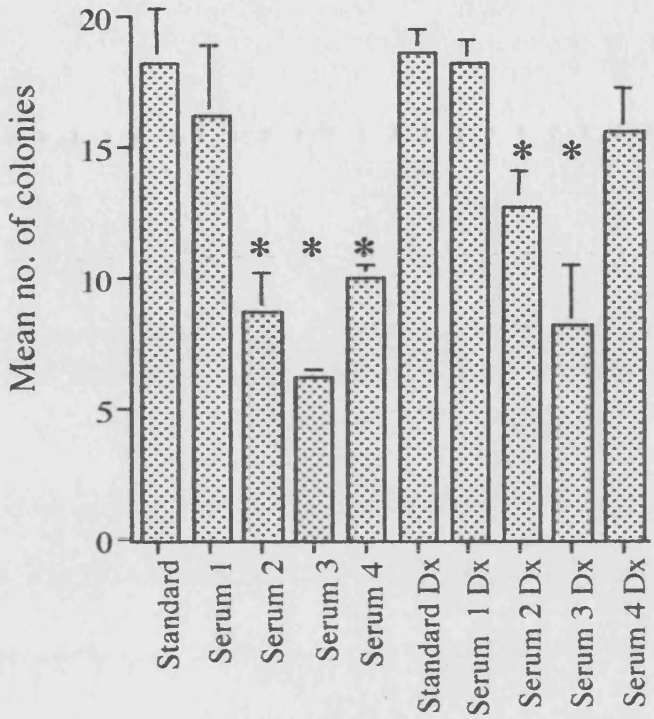
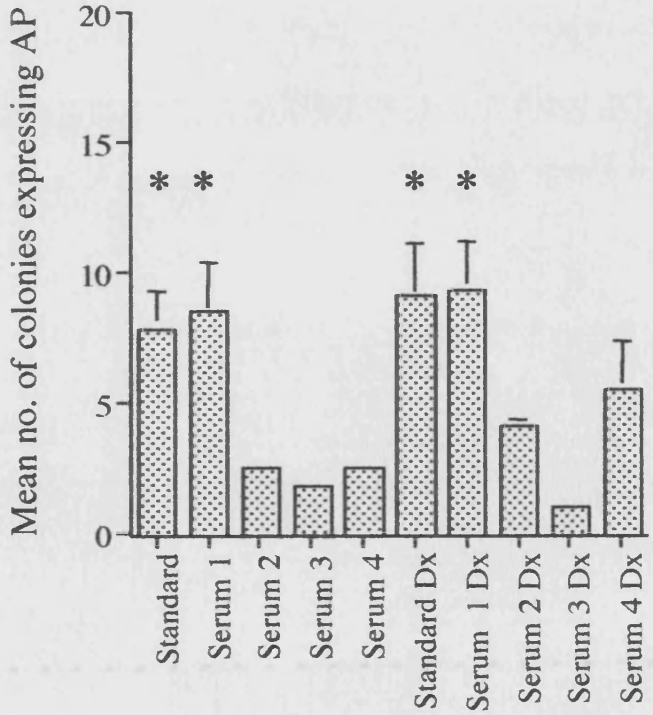


Figure 3.5: The Effect of Different Sera on (a) the Total Number of Colonies and (b) Number of AP+ Colonies.



(a)



(b)

Figure 3.6: The Effect of Different Sera on the Percentage of Colonies Expressing AP.

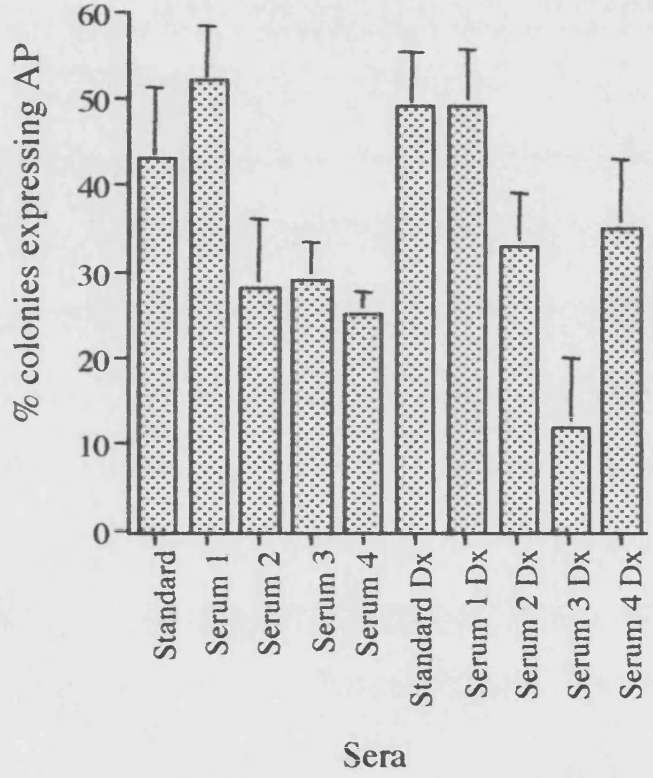
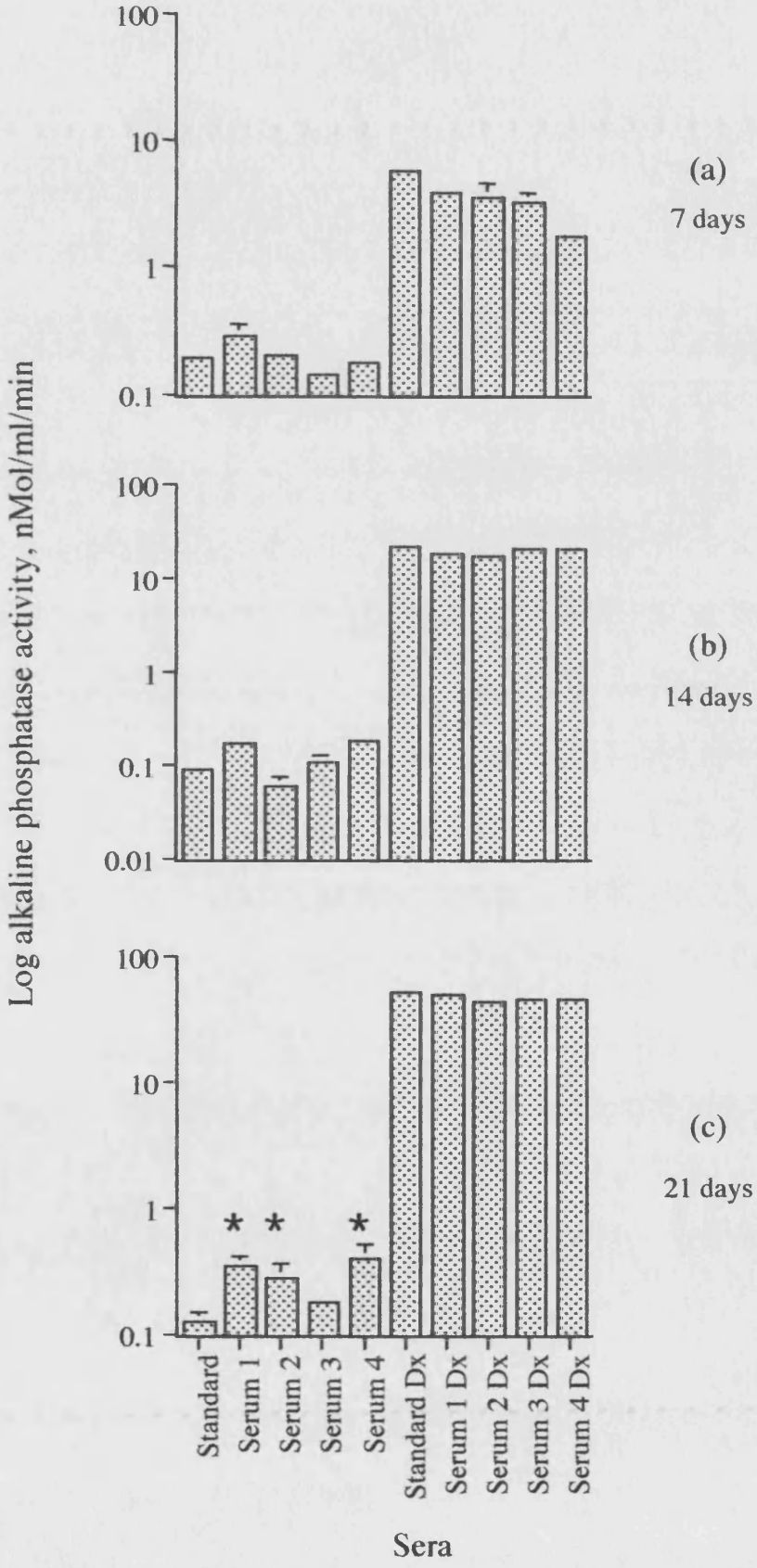


Figure 3.7: The Effect of Different Sera on Alkaline Phospahatase Activity of BMSC at (a) 7, (b) 14, and (c) 21 Days in Culture.



PPP Experiments.

Human platelet poor plasma (PPP) was prepared from blood donated by healthy donors as previously described, and compared, using multiple parameters, to the standard serum currently in use and known to support proliferation and osteogenic differentiation. The PPP was tested for its ability to support human marrow cells in culture by measuring the following parameters:

(i) Cell numbers
(ii) Cell proliferation
(iii) Colony forming efficiency (Total colonies and AP expressing)

Experiment PPP 1.

To assess the ability of PPP to support BMSC in culture the initial experiment compared media supplemented with 10% PPP (+/- Dx) with 10% FCS (+/- Dx) in its ability to support proliferation. Cells were derived as previously described, from a 68 year old female patient undergoing cardiothoracic surgery at Bristol's Frenchay Hospital. Cells were randomly assigned to 1 of 4 experimental groups (table 3.1). The cells were seeded at a density of $1x10^4/cm^2$ into 3 x T75 culture flasks/experimental group.

Table 3.1. Experiment PPP1 Treatment Groups.

Control	**PPP**
DMEM 90%	DMEM 90%
FCS Sera 10%	PPP Sera 10%
Asp 2 P (100μm)	Asp 2 P (100μm)
Control Dx	**PPP Dx**
DMEM 90%	DMEM 90%
FCS Sera 10%	PPP 10%
Asp 2 P (100μm)	Asp 2 P (100μm)
Dx 10^{-8} M	Dx 10^{-8} M

The cells were incubated at 37°C in 5% CO_2 for 7 days in a humidified atmosphere. At this point the media, containing non-adherent cells, was removed and the cells were replenished with appropriate fresh media twice weekly. The cultures were maintained for 3 weeks at which point the media was removed, the cells passaged and counted on a Coulter counter as previously described.

3.4: Results (PPP).

3.4.1: PPP1 Cell Numbers.

Cultures supplemented with a PPP±Dx. had significantly fewer cells ($p < 0.01$) than comparable cultures supplemented with FCS±Dx (fig. 3.8).

Experiment: PPP2.

To extend the previous experiment to assess the effects of higher concentrations of PPP, marrow stromal cells were isolated from the rib of a 55 year old male undergoing cardiothoracic surgery at Bristols Frenchay hospital. Cells were randomly assigned to 1 of 6 experimental groups (table 3.2) to compare media supplemented with 10% PPP (+/- Dx), or 30% PPP(+/- Dx) with 10% FCS (+/- Dx) in their ability to support osteogenic proliferation/differentiation.

Table 3.2. Experiment PPP2 Treatment Groups.

Control	**10% PPP**	**30% PPP**
DMEM 90%	DMEM 90%	DMEM 70%
FCS Sera 10%	PPP Sera 10%	PPP Sera 30%
Asp 2 P (100μm)	Asp 2 P (100μm)	Asp 2 P (100μm)
Control Dx	**10% PPP Dx**	**30% PPP Dx**
DMEM 90%	DMEM 90%	DMEM 70%
FCS Sera 10%	PPP 10%	PPP 30%
Asp 2 P (100μm)	Asp 2 P (100μm)	Asp 2 P (100μm)
Dx 10^{-8} M	Dex 10^{-8} M	Dx 10^{-8} M

The cells were seeded at a density of $1x10^4/cm^2$ into 4 x T75 culture flasks/experimental group to assess cell numbers and at $1x10^4/cm^2$ into 6 well plates (3 wells/group) to measure effects on proliferation and CFE.

The cells were incubated at 37°C in 5% CO_2 for 7 days in a humidified atmosphere. At this point the media, containing non-adherent cells, was removed and the cells were replenished with appropriate fresh media twice weekly. The cultures were maintained for 3 weeks at which point the media was removed, the cells to be assessed for cell numbers were passaged and counted on a Coulter counter as previously described. Colonies were visualised in the 6 well plates after staining with 1% (w/v) methylene blue in 10mM tris-borate buffer and AP expressing colonies were assessed after staining with Fast Red salt, both methods as described previously.

3.4.2: PPP 2 Cell Numbers.

Cultures supplemented with 10% and 30% PPP±Dx had significantly fewer cells ($p < 0.01$) than comparable cultures supplemented with FCS±Dx, (fig. 3.9).The ratio of cells in the -Dx cultures; FCS:PPP(10% and 30%) = 8.6. In +Dx cultures, cell ratios were FCS:10% PPP = 5.9; FCS:30% PPP = 17.75.

3.4.3: PPP 2 Colony Numbers.

Cultures supplemented with 10% and 30% PPP±Dx had significantly fewer colonies ($p < 0.01$) than comparable cultures supplemented with FCS±Dx (fig. 3.10a).
Although the control Dx colony numbers are less than the control group this does not reflect the amount of colony coverage. Dx colonies although less in number were generally of a much greater size.

No AP+ colonies were formed in PPP supplemented cultures (fig. 3.10b).

Experiment: PPP3.

The previous experiment (3.4.2) was repeated using different donors for both serum (male 25 years) and cells (female 55 years old). PPP concentrations of 1%, 5%, 10%, 20% and 30% (as formulated above) +/-Dx were used.

3.4.4: PPP 3 Cell Numbers.

Cultures supplemented with 10% and 30% PPP±Dx had significantly fewer cells ($p < 0.01$) than comparable cultures supplemented with FCS±Dx (fig. 3.11). A negative correlation was seen between PPP concentration and cell numbers (fig. 3.12a,b). -Dx; adj. $r^2 = -0.91$; $p < 0.01$: +Dx; adj. $r^2 = -0.71$; $p < 0.05$.

3.4.5: PPP 3 Colony Numbers.

PPP supplemented cultures had significantly fewer colonies, and fewer AP+ colonies either with or without the addition of Dx (fig. 3.13).

Experiment: PPP 4.

The above experiments were again repeated using different donors for both serum (male 25) and cells (male 57 years old). Additionally, the PPP was supplemented with EGF at 3.0 ng/ml, IGF1 at 3.0 ng/ml and PDGF(bb) at 5ng/ml (PPP+). Previous work has shown that in serum free systems, these components were necessary to stimulate cell proliferation[227,228]. These supplements are also reported to support the proliferation and osteogenic differentiation of osteogenic stem cells *in vitro* [88,96,97,103,158,229].

3.4.6: PPP 4_ Cell Numbers. and Colonies

In this experiment cells, in PPP supplemented cultures almost totally failed to adhere and this resulted in an undetectable level of cell numbers (fig. 3.14) and colonies (fig. 3.15).

Figure 3.8: The Effect of Platelet Poor Human Plasma on BMSC Numbers: Experiment I.

* $p = < 0.01$

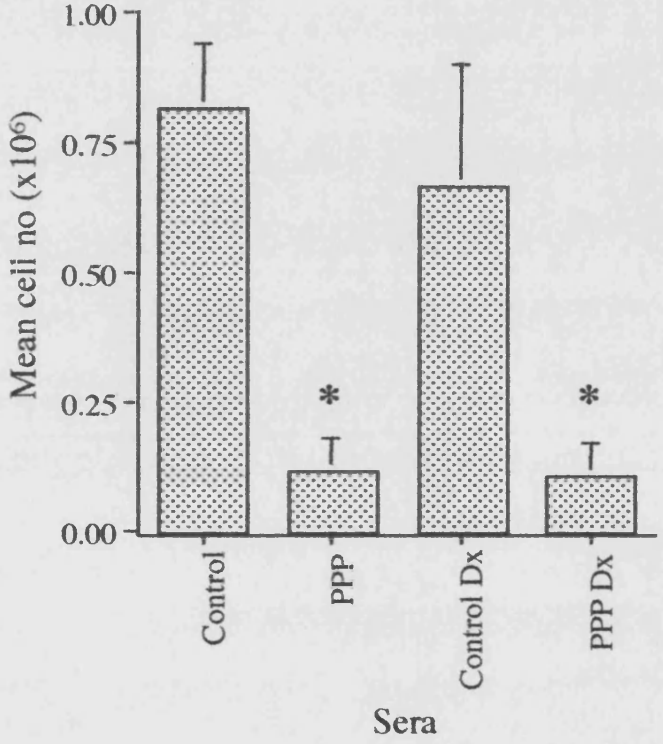


Figure 3.9: The Effect of Platelet Poor Human Plasma on BMSC Numbers: Experiment II.

* p = < 0.01

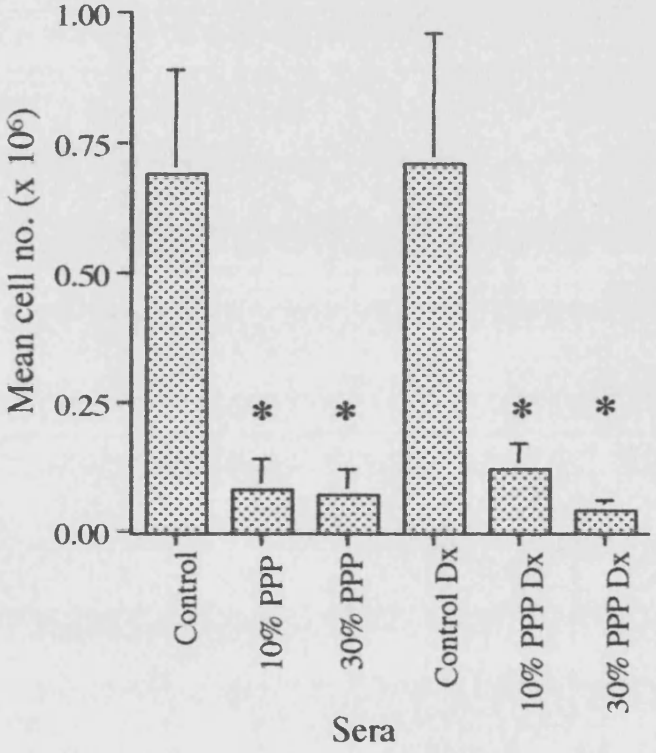


Figure 3.10: The Effect Platelet Poor Human Plasma on (a) the Total Number of Colonies and (b) the Number of AP+ Colonies. II.

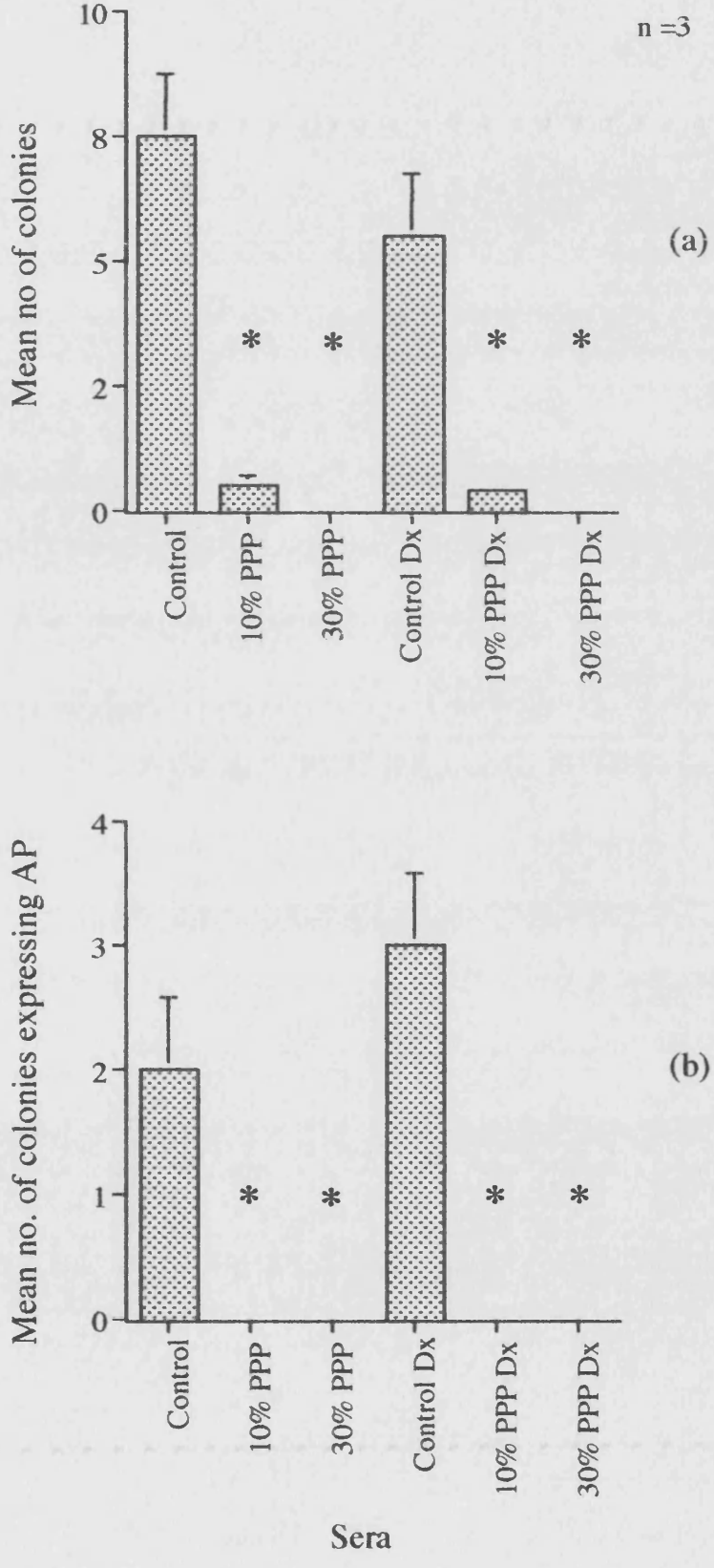


Figure 3.11: The Effect of Platelet Poor Human Plasma on BMSC Numbers: Experiment I III.

* p = < 0.01

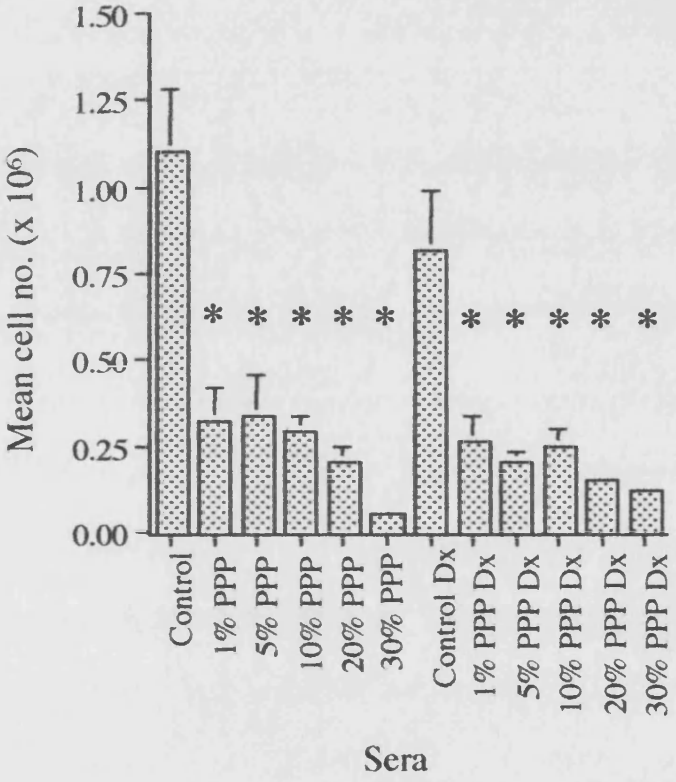
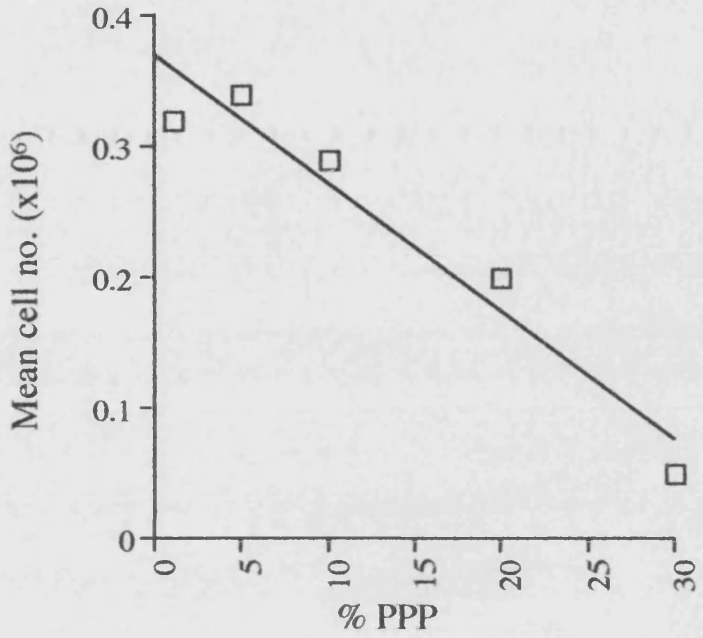
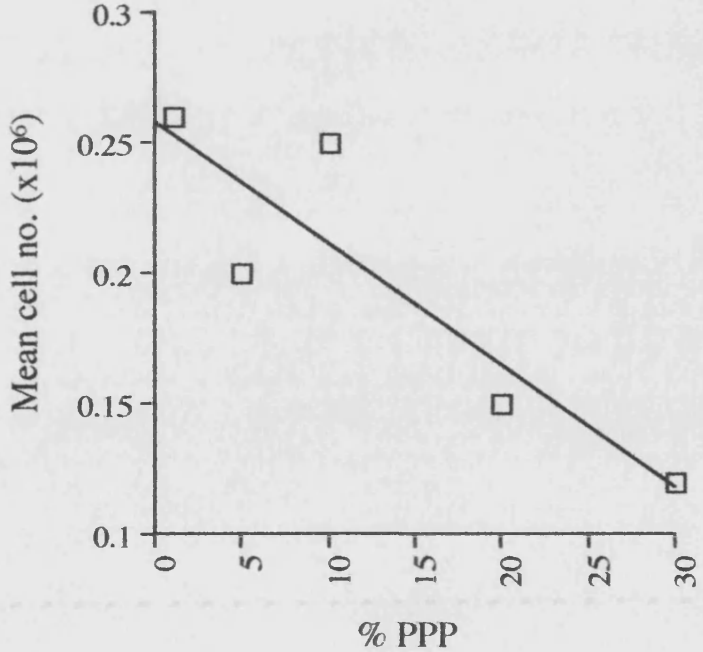


Figure 3.12:Relationship between % PPP and Cell Number (a) -Dx, (b) +Dx.



Adj. $R^2 = 0.91$ $P < 0.01$



Adj. $R^2 = 0.73$ $P < 0.05$

Figure 3.13: The Effect of Platelet Poor Human Plasma on the (a) Total Colony Number and (b) AP Expressing Colony Number: III.

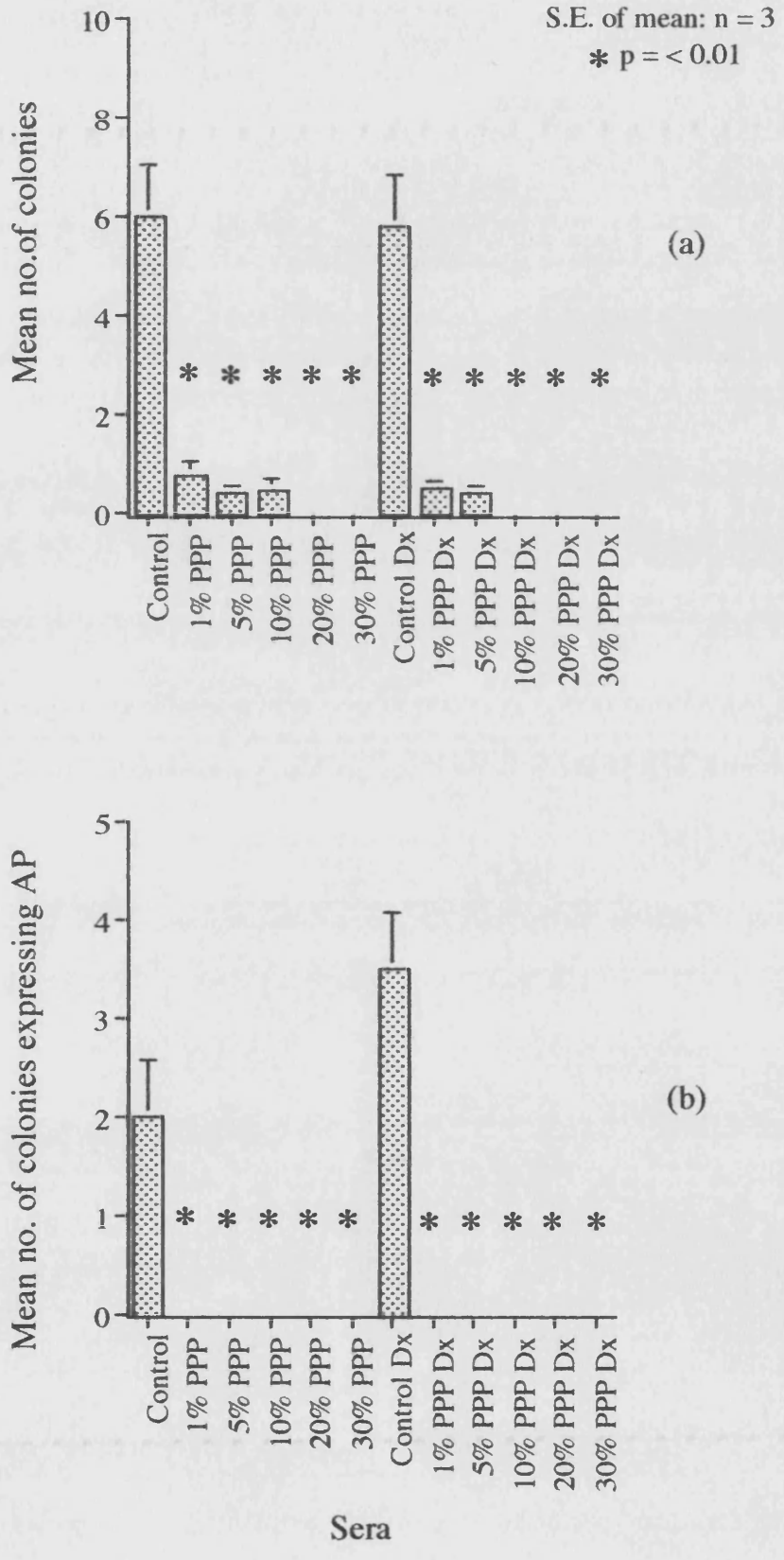


Figure 3.14: The Effect of Platelet Poor Human Plasma Supplemented with EGF, PDGFbb and IGF-1 on the Proliferation of Human BMSC.

* p = < 0.01

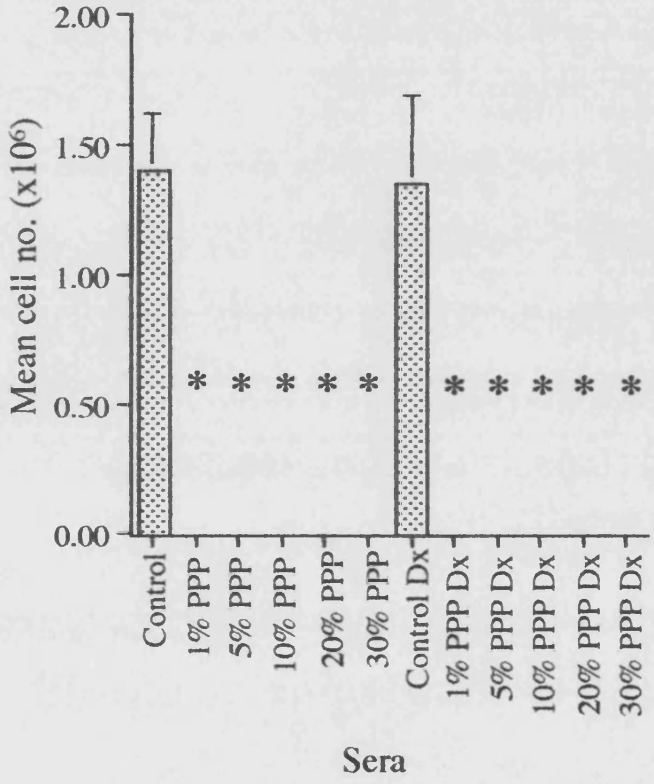
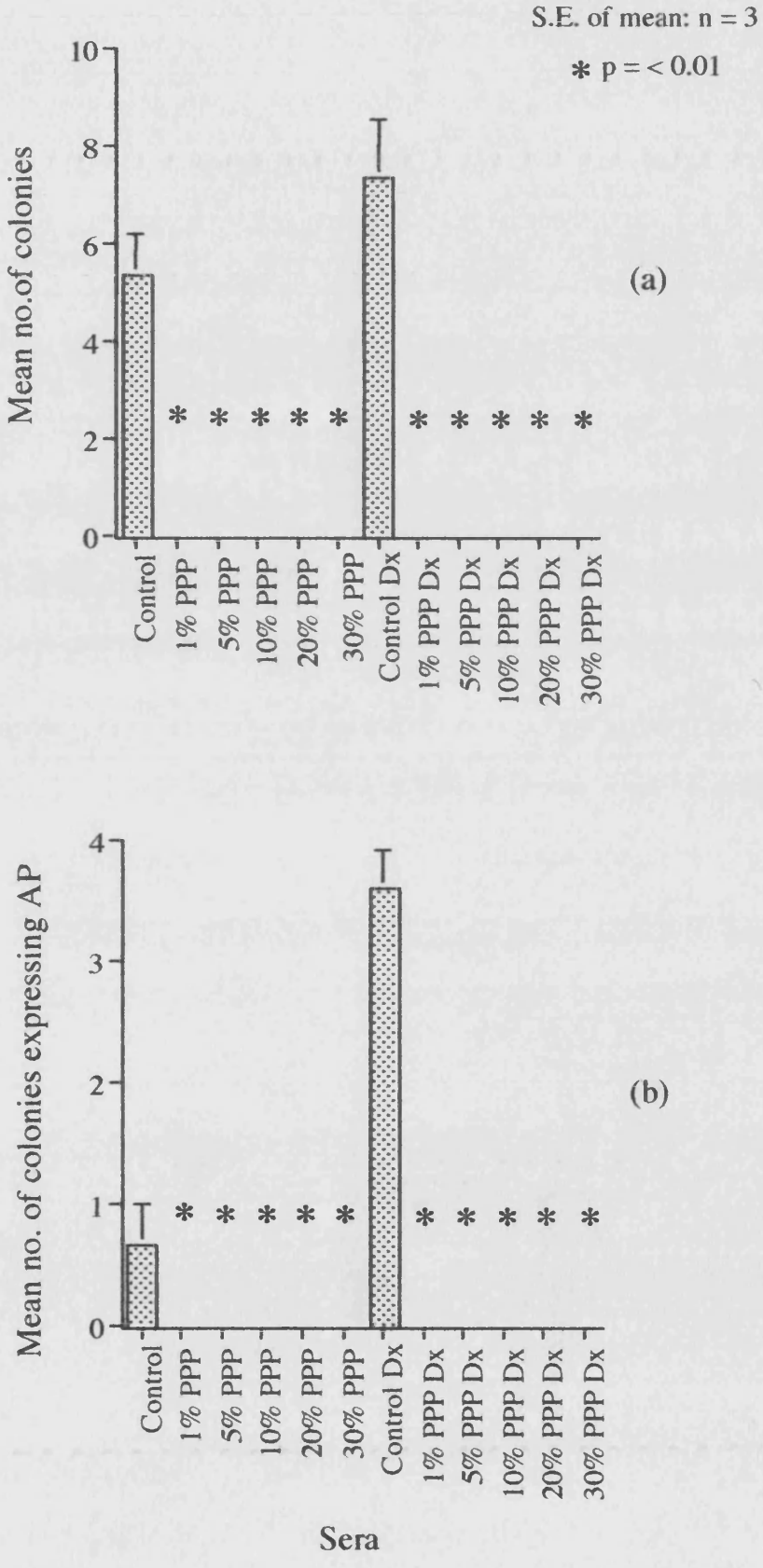


Figure 3.15: The Effect of Platelet Poor Human Plasma Supplemented with EGF, PDGFbb and IGF-1 on the (a) Total Colony Number and (b) AP Expressing Colony Number.



3.5: Discussion.

Supplementation of media preparations with serum is an absolute requirement for the osteogenic differentiation of cells in culture. Different batches of sera reflect wide variations in the ability to support this process, and accordingly, choice of sera should be regarded as a prime importance. To maintain some consistency over a range of studies, it is therefore essential that a suitable batch is identified and purchased in sufficient volume to provide supplementation for a number of studies over several years.

3.5.1: Human Platelet Poor Plasma.

Samples of human PPP were obtained from a number of healthy volunteers and were compared using multiple parameters to assess its ability to support proliferation and osteogenic differentiation. It was found that at all concentrations of PPP used, either with or without additional supplements, PPP was consistently inferior to FCS in all measured parameters. It was considered that the poor performance of this supplement may have been related to the method of preparation. To test this, the preparation protocol was modified to include a final step of heat inactivation: 56°C for 30 minutes. This modification did not significantly alter the results (not shown) and PPP was therefore not considered as a media supplement for this study.

The inability of PPP to support BMSC in culture was primarily related to a failure of cell adherence, secondary to a low proliferation rate in those that had attached.
When one considers the importance of platelet derived growth factors in bone cell cultures,[103,230] the inability of PPP without supplementation (PPP-) of growth factors, to support BMSC in culture is not surprising. Studies have shown that PDGF and EGF are absolute requirements for CFU-F colony development in cultures of BMSC[229]. The addition of neutralising antibodies to PDGF and TGFβ has been shown to cause profound inhibition of colony formation in a similar system[231].

In the PPP 4 experiment, the failure of the added growth factors to enhance cell adherence might suggest that the positive effect of these factors is probably not related to the promotion of uncommitted non-adherent cells, it has been suggested that the positive effects of these agents are related to the stimulation of initial proliferation[231]. It should be noted however, that in this case, none of the PPP supplemented cultures had any detectable numbers of cells or colonies. Comparing these findings with the other experiments strongly suggests that the PPP is the cause of the deficit and it may be that significant inter-donor differences in the ability to support cells in culture are to blame.

It would be interesting to expand on these findings and investigate the effect of other platelet derived growth factors such as TGFβ, on this system. TGFβ stimulates colony formation,[232] and it has been proposed that its presence in combination with other factors is essential for the initial proliferation of colony forming cells[231]. It is possible that the inclusion TGFβ, or other factors, to a PPP system might produce a serum capable of supporting BMSC in culture but the overall aims of this study do not warrant any further investigation in that direction.
However, the purpose of this study was to identify a serum capable of supporting the proliferation and osteogenic differentiation of BMSC in culture, and this has been achieved.

3.5.2: FCS.

Samples of four batches of FCS were compared using multiple parameters to assess their ability to support proliferation and osteogenic differentiation. Serum 1, Globepharm 2704, was selected as the supplement for use in this study for a number of reasons: (i) No other serum was significantly better at supporting cell proliferation, either with or without the addition of Dx. (ii) The Globepharm serum was significantly better than the others tested in its ability to support CFE for both total colonies and AP expressing colonies, either with or without the addition of Dx. (iii) The number of AP colonies, expressed as a percentage of the total colonies, was greatest in cultures supplemented with Globepharm 2704, either with or without the addition of Dx. (iv) AP activity was not significantly higher at any time point, in any other sera tested, either with or without the addition of Dx, than the Globepharm serum. Accordingly the Globepharm batch 2704 was acquired for use in subsequent *in vitro* studies.

3.6: Summary.

FCS and PPP were investigated using multiple parameters in order to assess their ability to support BMSC in culture. Cultures supplemented with FCS from different sources displayed a wide range of ability to support BMSC in culture. All FCS supplemented cultures were superior to PPP supplemented in every parameter measured and the most effective FCS serum (Globepharm 2704) was chosen.

Chapter 4: The *In Vitro* Effects of TGFβ and FGF-1 Treatments on Bone Marrow Stromal Cells.

4.1: Introduction.

FGFs and members of the TGF-β superfamily are important regulators of bone formation during development and in the adult skeleton[54,70,233-235]. Both of these factors are produced by cells of the osteoblast lineage *in vivo* and *in vitro* [182,236,237] and are found in significant quantities in the mineralised bone matrix[238]. Numerous studies have investigated the effects of these factors on bone cell cultures *in vitro* (for TGFβ review see Bonewald[239] for FGF see Hurley[240]). Most studies involving FGF have been performed only during the proliferative phase of osteoblast development[241] and likewise, few studies have investigated the temporal application of TGFβ in this type of system. In the present investigation, the effects of FGF-2 (2.5ng/ml) and TGF-β (250pg/ml), on the proliferation and differentiation of osteogenic precursors in BMSC were studied

These cultures are initiated by a small number of primitive clonogenic cells (CFU-F) that must first become adherent before giving rise to progeny capable of proliferation and further differentiation. To determine the temporal effects of TGFβ-1 and FGF-2 on each of the processes (adherence, proliferation and differentiation) the factors were added at different stages of culture cycle and for different periods viz days 0-28 (continuous) 0-15 (early) and 15-28 (late).

4.2: Methods.

Bone marrow cells were obtained as described previously, from segments of rib removed during routine thoracic surgery from a female patient aged 69 at Frenchay Hospital, Bristol. The cells were plated at a density of 2 x 10^4/cm in 75 cm^2 flasks (4 flasks/experimental group) or petri dishes (4 petris/experimental group) containing DMEM supplemented with 10% FCS and 100μM ascorbate-2-phosphate (control) or the same medium supplemented with 2.5 ng/ml FGF-2, 250pg/ml TGFβ–1 or both factors in combination. (Table 4.1). The medium was replenished at 7 days and thereafter twice weekly. After a total of 24 days in culture the following parameters were determined;

(1) Total colony number.
(2) Total colony area.
(3) Mean colony area.
(4) AP positive colonies.
(5) Total number of cells.
(6) Proportion of STRO-1 and AP positive cells (Flow cytometry).

Colony number

The cultures were fixed in methanol (5 mins) and then stained with a solution of 1% Methylene blue /ddH2O for 30 minutes. n= 4 for each treatment.

Proportion of AP+ Colonies.

The cultures were fixed in methanol and then stained for AP activity using Napthol AS-MX as substrate and Fast Red as the coupling dye. Incubation with the substrate was at room temperature @ pH9.2. (n= 4).

Cell numbers.

Following sequential treatment with collagenase/trypsin-EDTA, cells were counted on a Coulter electronic particle counter model ZF. (n= 4).

Colony areas.

Colony areas were assessed manually using the image analysis software package Image v 1.6.1.

Flow Cytometry.

Binding of the monoclonal antibodies, STRO-1 and B4-78 (AP) was determined as described in Chapter 2 and according to the method of Gronthos et.al. [242] .

Statistical Analysis.

All experiments were repeated at least once and data from a single representative experiment is shown. The distribution of the data was confirmed as normal using the Shapiro Wilk W test, where data was not normally distributed, a log transformation was used to normalise the distribution prior to test. Differences in the data were assessed using the one way Analysis of Variance followed by the Tukey-Kramer test of Honest Significant Difference ($p < 0.05$).

Table 4.1: Experimental Groups.

	Treatment Schedule		
Group	**Day 0**	**Day 15**	**Day 28**
1. Control	Control	Control	Control
2. Control-FGF	Control	FGF-2	FGF-2
3. Control-TGF	Control	TGF	TGF
4. Control-Both	Control	FGF-2/TGF	FGF-2/TGF
5. FGF	FGF-2	FGF-2	FGF-2
6. FGF-TGF	FGF-2	TGF	TGF
7. FGF-Control	FGF-2	Control	Control
8. FGF-Both	FGF-2	FGF-2/TGF	FGF-2/TGF
9. TGF	TGF	TGF	TGF
10. TGF-FGF	TGF	FGF-2	FGF-2
11. TGF-Both	TGF	FGF-2/TGF	FGF-2/TGF
12. TGF-Control	TGF	Control	Control
13. Both	FGF-2/TGF	FGF-2/TGF	FGF-2/TGF
14. Both-Control	FGF-2/TGF	Control	Control
15. Both-FGF	FGF-2/TGF	FGF-2	FGF-2
16. Both- TGF	FGF-2/TGF	TGF	TGF

Figure 4.1: Effect of Growth Factor Treatment on Total Colony Numbers.

Error bars represent
S.E. of mean: n = 4

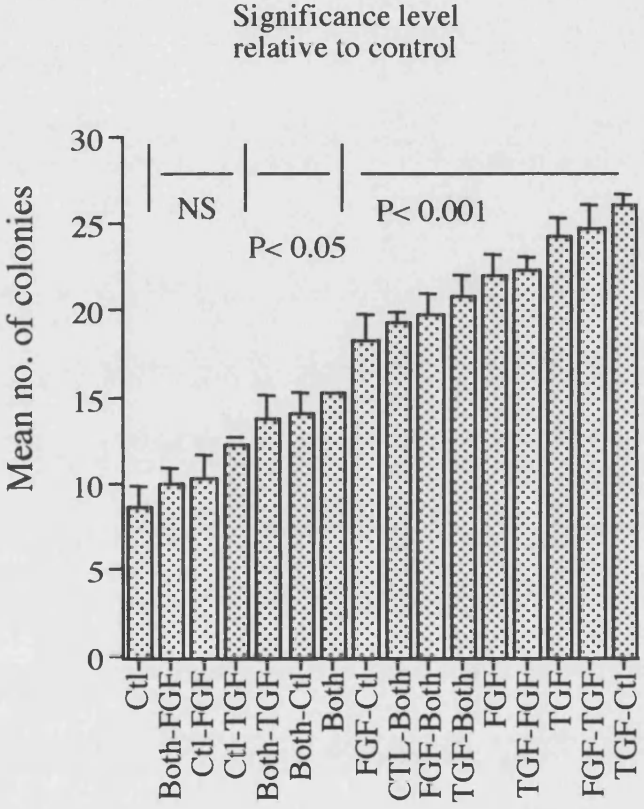


Figure 4.2 (a-e)

Each image shows 4 repeats per experimental group. Colonies are stained with methylene blue and appear as blue regions within the petris dishes. Images are presented in ascending rank order of colony numbers.

(a) Control: Cultures where no growth factors where added to the culture medium for entire 28 days.

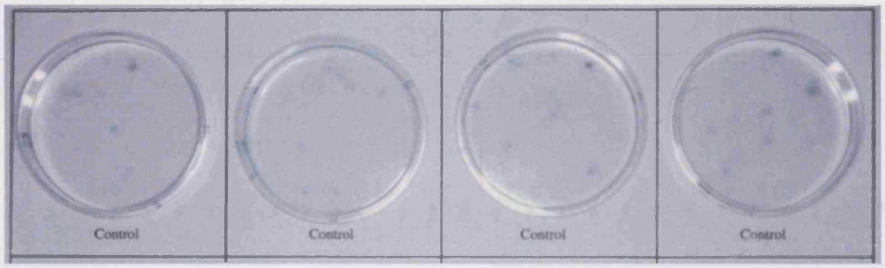
(b) Both-FGF: Cultures treated with TGFβ-1 and FGF-2 supplemented medium for first 15 days then with FGF-2 only from days 15 - 28.

(c) Control-FGF: No growth factors added to the culture medium for first 15 days then treated with FGF-2 only from days 15 - 28.

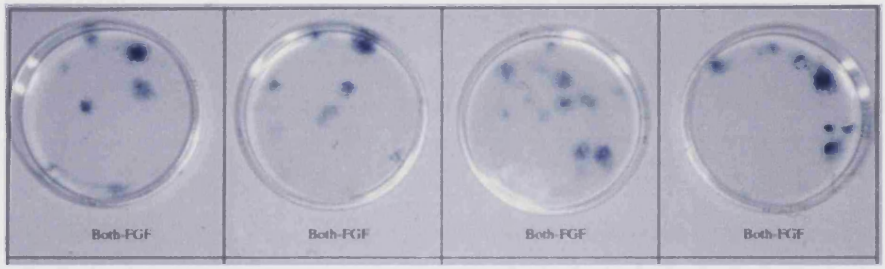
(d) Control-TGFβ: No growth factors added to the culture medium for first 15 days then treated with TGFβ-1 only from days 15 - 28.

(e) Both-TGFβ: Cultures treated with TGFβ and FGF-2 supplemented medium for first 15 days then treated with TGF-1 only from days 15 - 28.

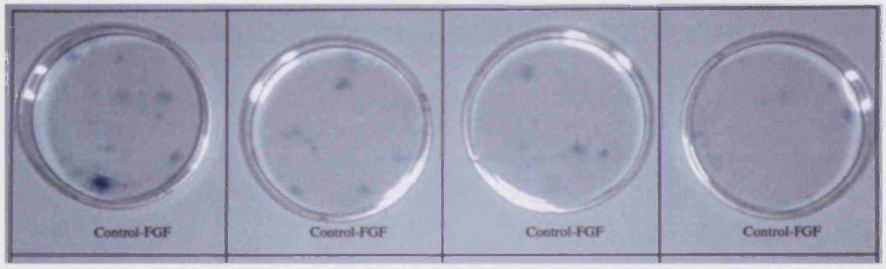
Figure 4.2 (a-e): Effect of Treatment on Total Colony Numbers.



(a)



(b)



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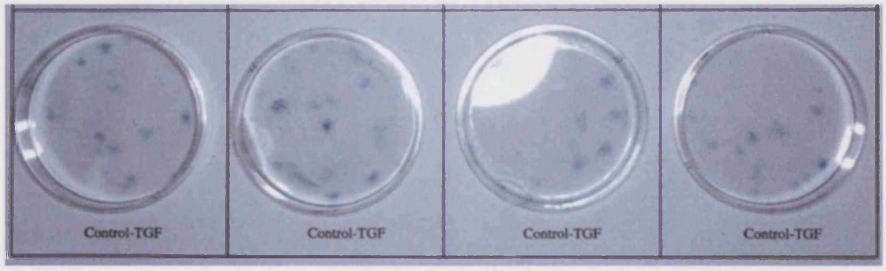

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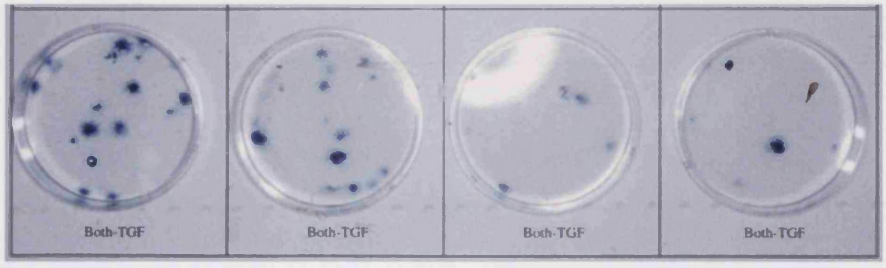


(e)

Figure 4.2 (f-j)

(f) Both-Control : Cultures treated with TGFβ-1 and FGF-2 supplemented medium for first 15 days then treated with standard medium from days 15 - 28.

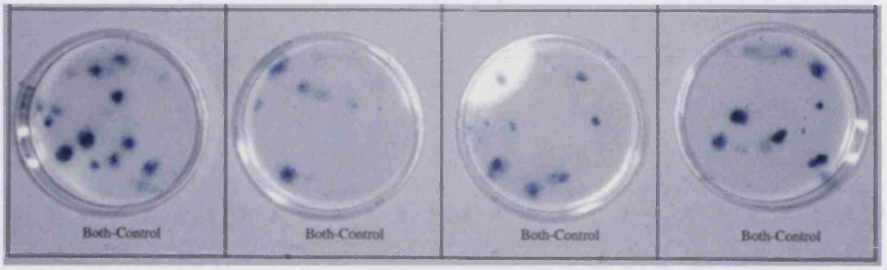
(g) Both: Cultures treated with TGFβ-1 and FGF-2 supplemented medium for entire 28 days.

(h) FGF-Control: Cultures treated with FGF-2 supplemented medium for first 15 days then treated with standard medium from days 15 - 28.

(i) Control-Both: No growth factors added to the culture medium for first 15 days then treated with FGF-2 and TGFβ-1 from days 15 - 28.

(j) FGF-Both: Cultures treated with FGF-2 supplemented medium for first 15 days followed by FGF-2 and TGFβ–1 treatment from days 15-28.

Figure 4.2 (f-j): Effect of Treatment on Total Colony Numbers



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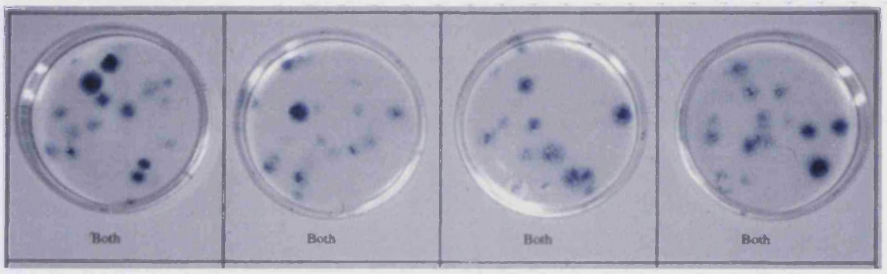
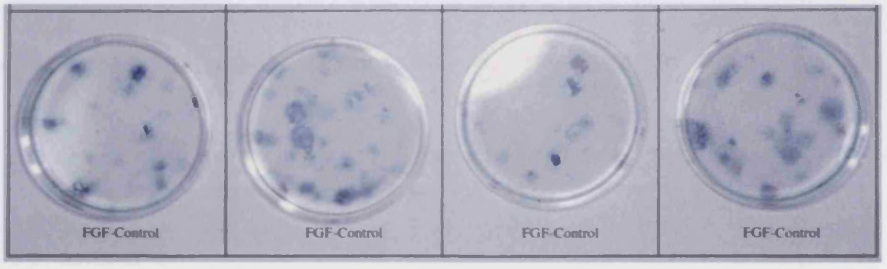


(g)



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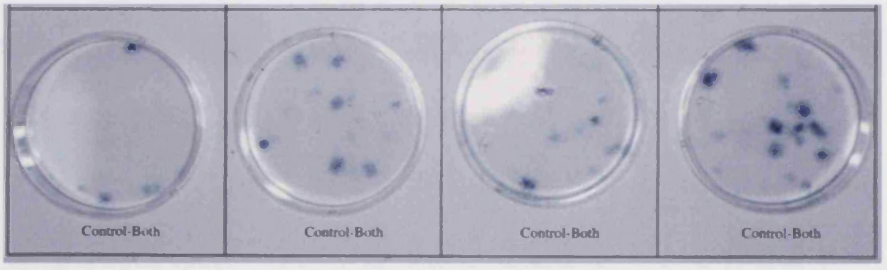
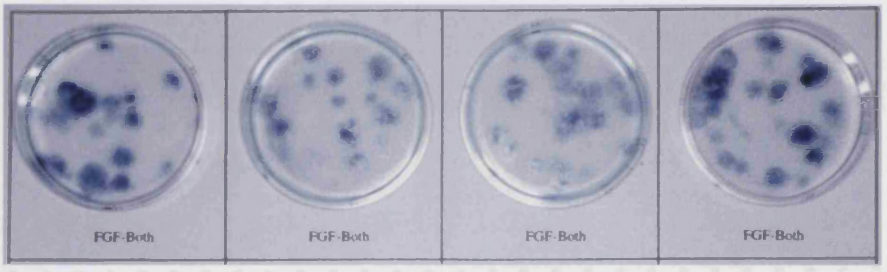


(i)



(j)

Figure 4.2 (k-p)

(k) TGF-Both: Cultures treated with TGFβ-1 supplemented medium for first 15 days followed by FGF-2 and TGFβ-1 treatment from days 15-28.

(l) FGF: Cultures treated with FGF-2 supplemented medium for entire 28 days.

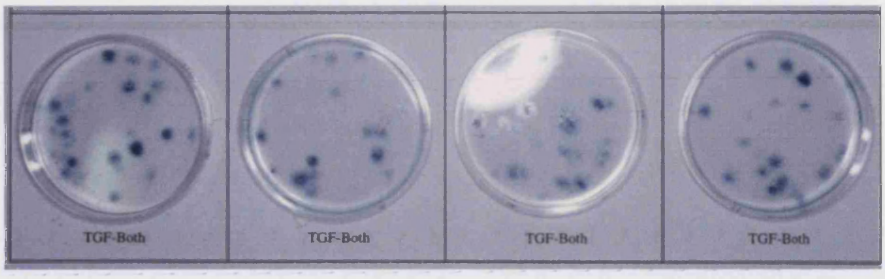
(m) TGF-FGF: Cultures treated with TGFβ-1 supplemented medium for first 15 days followed by FGF-2 treatment from days 15 - 28.

(n) TGF: Cultures treated with TGFβ-1 supplemented medium for entire 28 days.

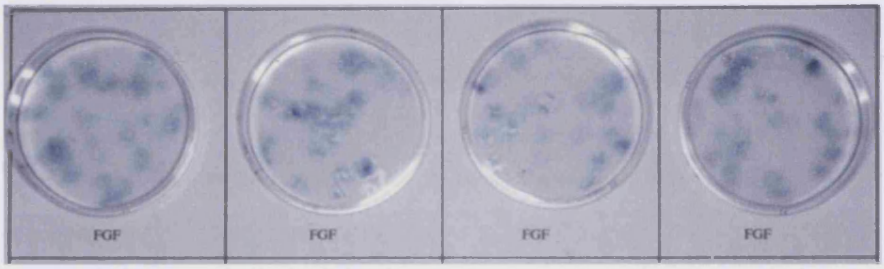
(o) FGF-TGF: Cultures treated with FGF supplemented medium for first 15 days followed by TGFβ-1 treatment from days 15 - 28.

(p) TGF-Control: Cultures treated with TGFβ-1 supplemented medium for first 15 days then treated with standard medium from days 15 - 28.

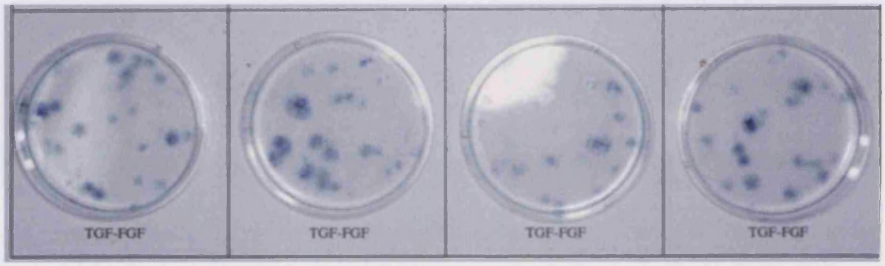
Figure 4.2 (k-p): Effect of Treatment on Total Colony Numbers



(k)



(l)



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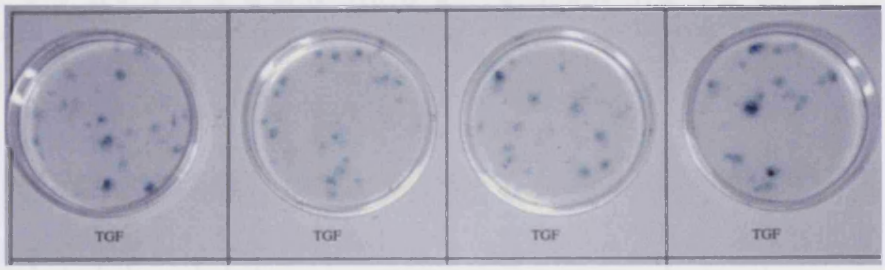

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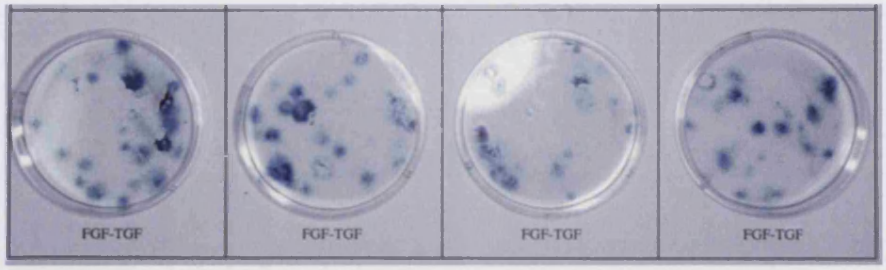
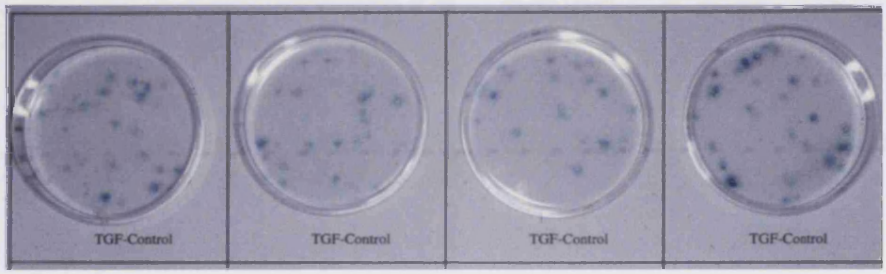


(o)



(p)

Figure 4.3 (a-d)

Each image shows 4 repeats per experimental group. TNSAP colonies were stained with Fast Red TR. Colonies in these groups were sparse extremely small and are indicated by arrows where appropriate. In some dishes white reflective areas representing unstained (AP -) colonies (e.g. arrow in b) can be seen. Except for the control, only groups having AP +ve colonies are shown. Images are presented in ascending rank order of colony numbers.

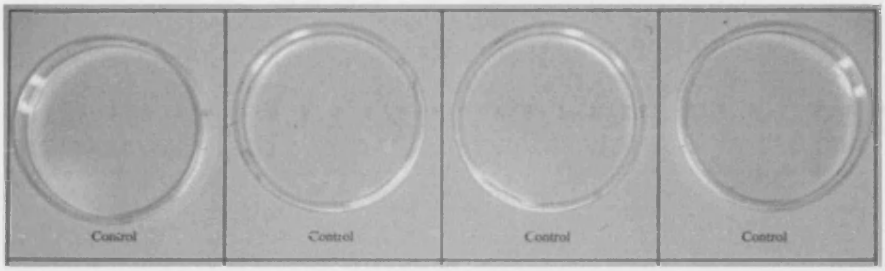
(a) Control: No growth factors added to the culture medium for entire 28 days. No AP +ve colonies were detected in this group.

(b) Both-FGF: Cultures treated with TGFβ–1 and FGF-2 supplemented medium for first 15 days then with FGF-2 only from days 15-28. Although no AP are reproduced in this image, one small AP +ve colony was detected by naked eye examination.

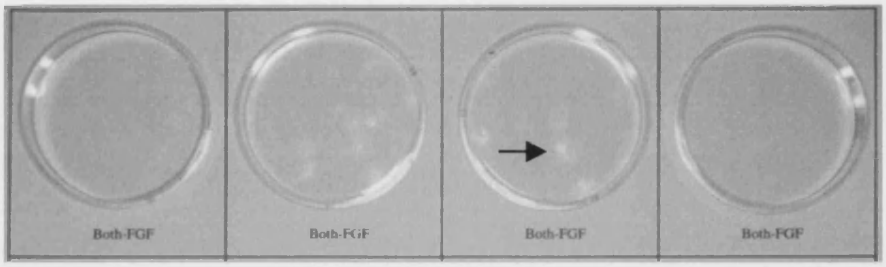
(c) Control-Both: No growth factors added to the culture medium for first 15 days then treated with FGF-2 and TGFβ–1 from days 15-28. One small AP colony was discovered and is illustrated (arrow).

(d) Both-TGFβ: Cultures treated with TGFβ–1 and FGF-2 supplemented medium for first 15 days then treated with TGF β -1 only from days 15 - 28. Two small AP+ve colonies were detected and are illustrated (arrows).

Figure 4.3 (a-d): Effect of Treatment on AP+ Colony Numbers.



(a)



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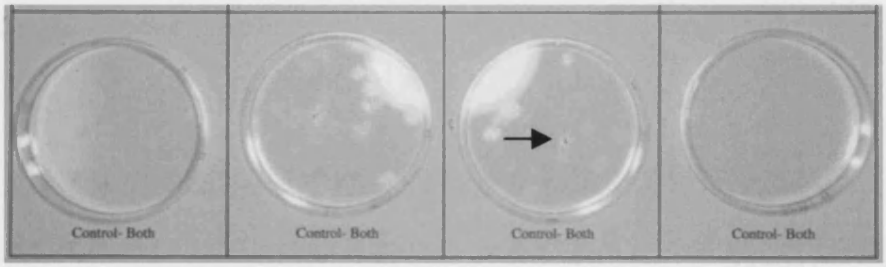
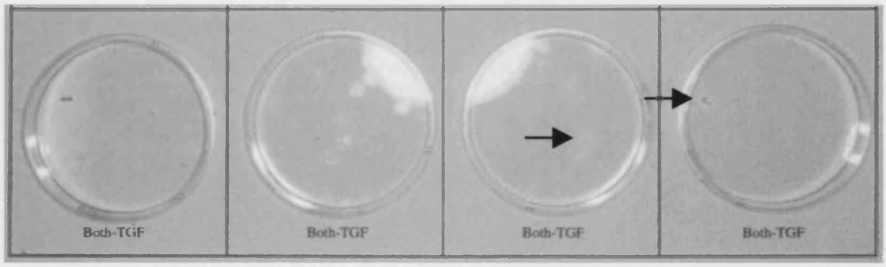


(c)



(d)

Figure 4.3 (e-h)

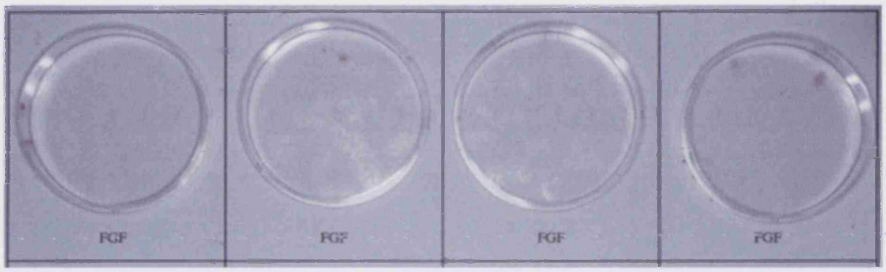
(e) FGF: Cultures treated with FGF-2 supplemented medium for entire 28 days.

(f) FGF-Both: Cultures treated with FGF-2 supplemented medium for first 15 days followed by FGF-2 and TGFβ treatment from days 15 - 28.

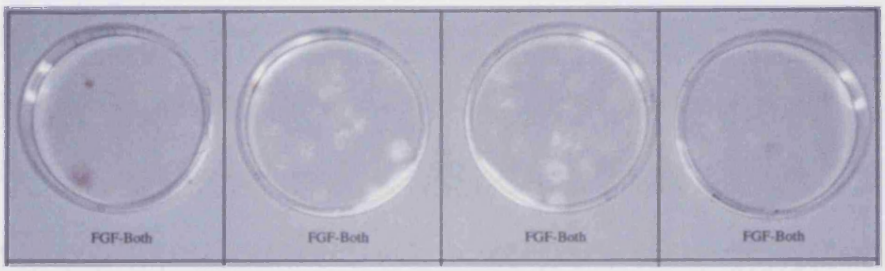
(g) FGF-Both: Cultures treated with FGF supplemented medium for first 15 days followed by TGFβ treatment from days 15 -28.

(h) FGF-Control: Cultures treated with FGF-2 supplemented medium for first 15 days then treated with standard medium from days 15 - 28.

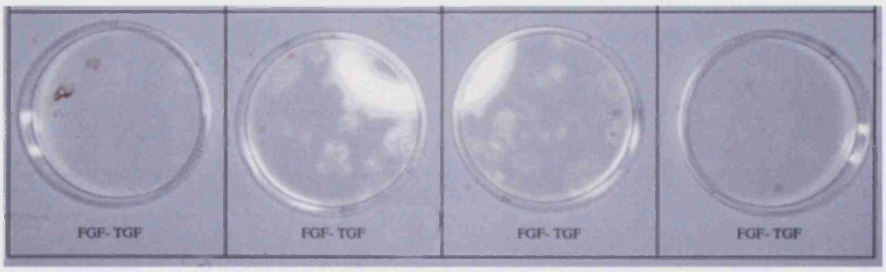
Figure 4.3 (e-h): Effect of Treatment on AP+ Colony Numbers.



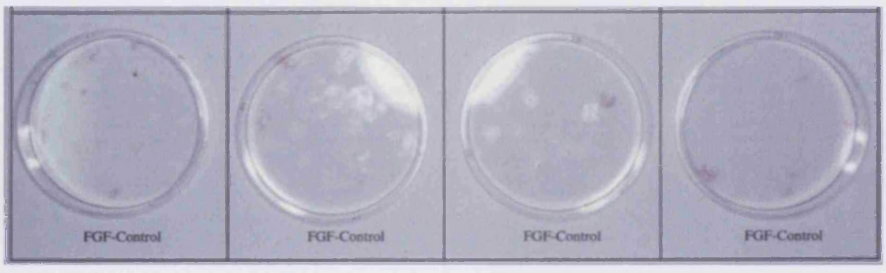
(e)



(f)



(g)



(h)

Figure 4.4: Effect of Treatment on Cell Number

Unless otherwise stated error bars represent S.E. of mean where n = 4

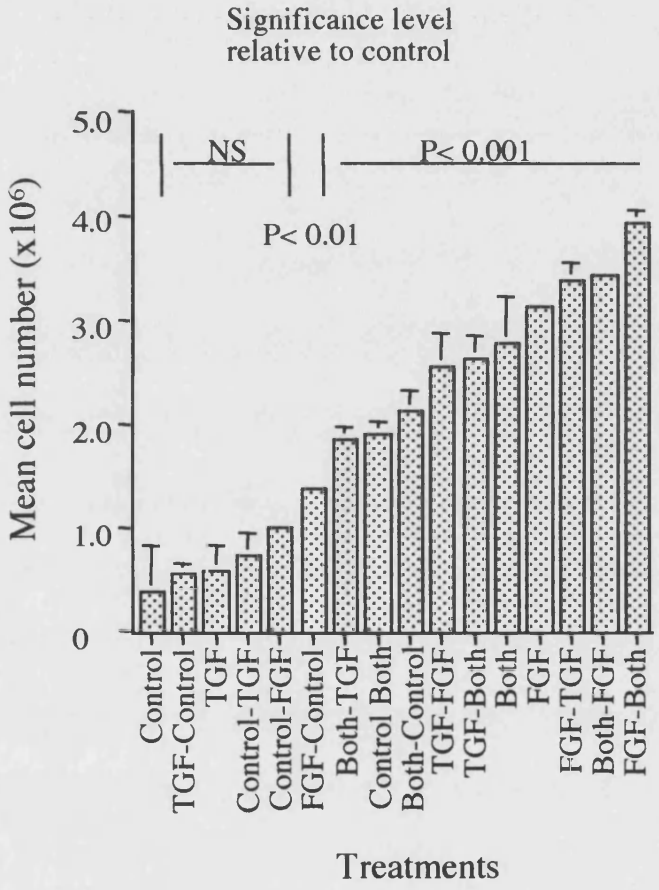


Figure 4.5: Effect of Treatment on the (a) Number and (b) Proportion of AP Colonies.

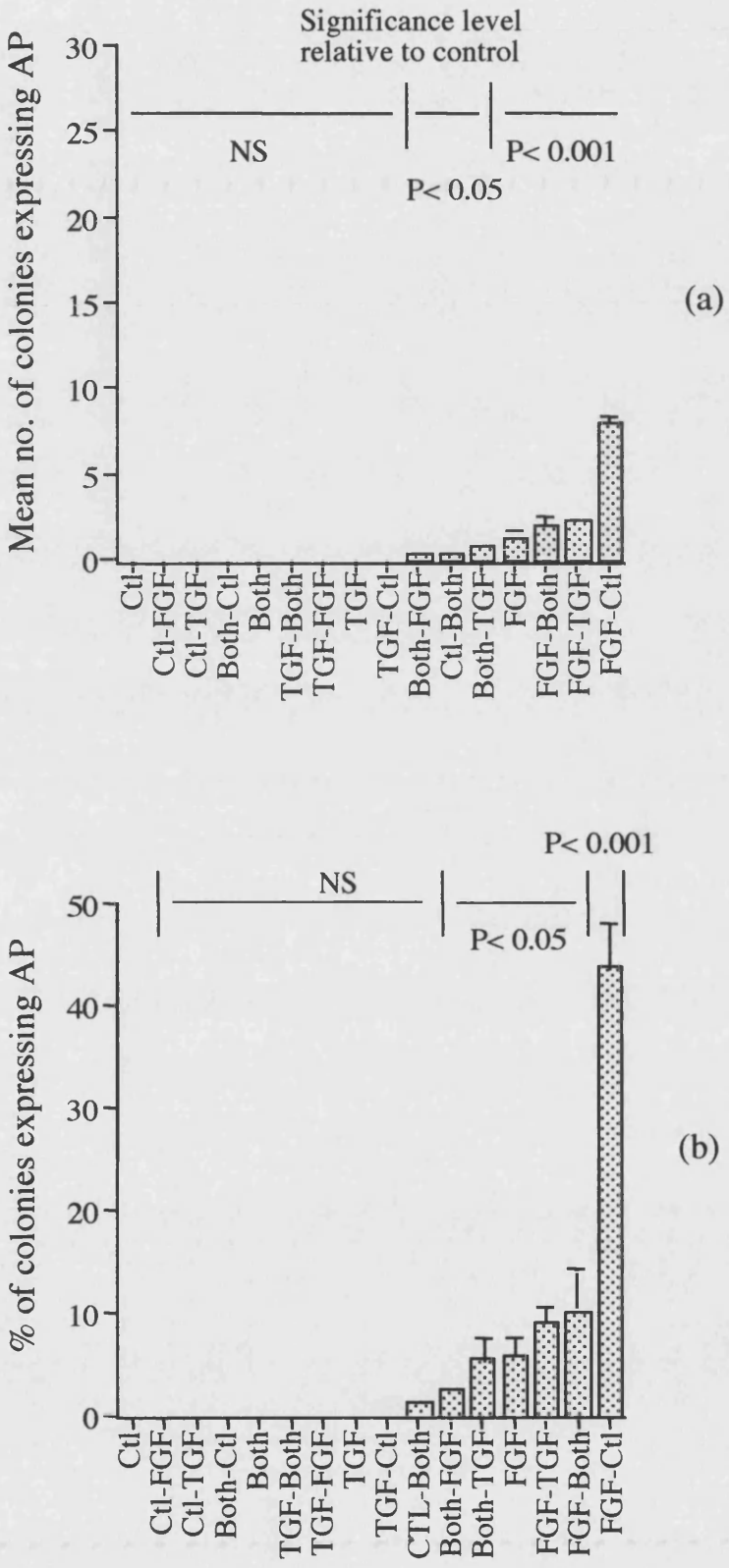


Figure 4.6: Proportions of Cells Expressing (a) STRO-1; (b) AP or (c) Both in Combination.

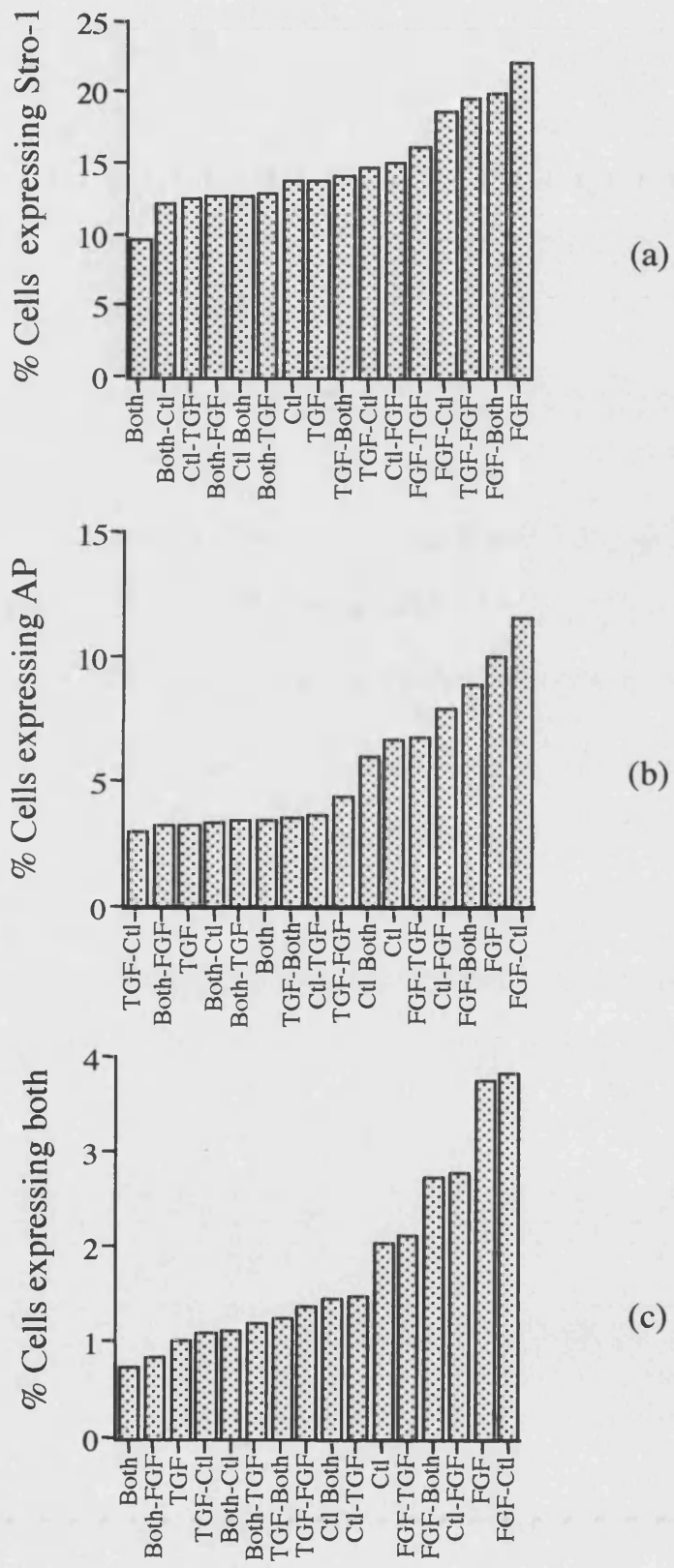


Figure 4.7:Effect of Treatment on (a) Mean Colony Area and (b) Total Colony Area.

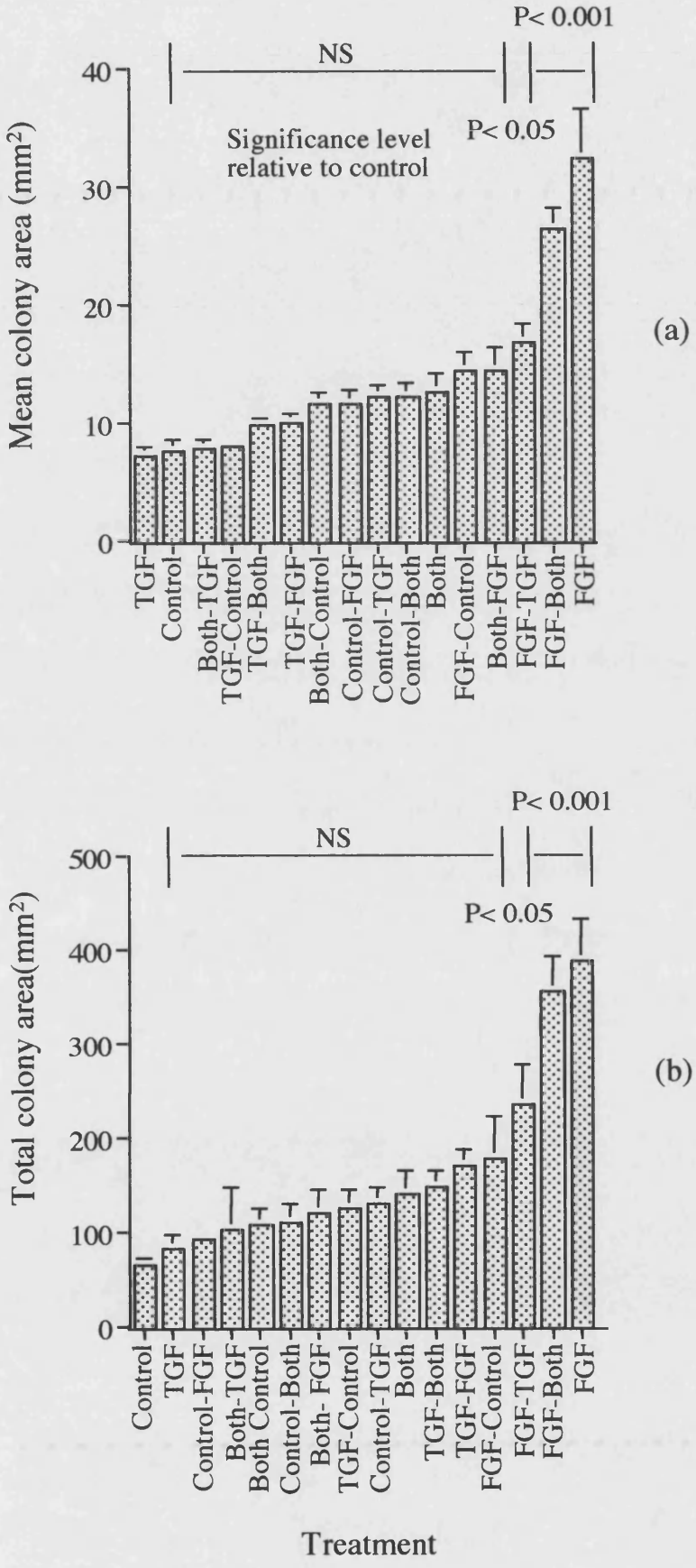
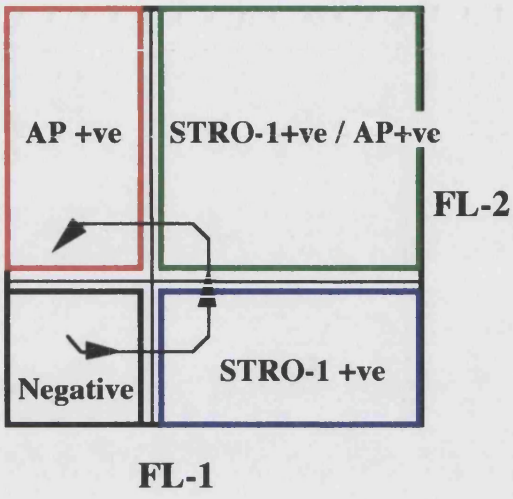


Figure 4.8: Flow Cytometry: Key to Populations.



Putative Differentiation Pathway

?	STRO-1, -ve / AP, -ve
Multipotential Precursor	STRO-1, +ve / AP, -ve
Committed Osteoprogenitor	STRO-1, +ve / AP, +ve
Maturing Osteoblast	STRO-1, -ve / AP, +ve

4.3: Results.

4.3.1: Colony Number.

Both TGF-β and FGF when applied singly and continuously, increased the total number of colonies formed by a similar amount (figs. 4.1; 4.2). To be effective, the factors had to be present early. The greatest increase was seen in cultures were TGFβ was applied early.

4.3.2: AP+ Colony Number.

In all cultures in which FGF was present early (0-7d), there was an increase in the number (figs. 4.3, 4.5a) and proportion of alkaline phosphatase-positive (AP+) colonies obtained (figs 4.5b). The effect was greatest in cultures treated with FGF for days 0-7 only.

4.3.3: Cell Numbers.

Treatment with FGF increased the number of cells in this system, particularly when applied early (fig.4.4). TGFβ had no effect on cell numbers in this system.

4.3.4: Flow Cytometry.

Analysis of the harvested cells by flow cytometry (fig. 4.6) using the B4-78 monoclonal antibody revealed that the effect of FGF was associated with an increase in the proportion of AP+ cells in both the STRO-1+/AP+ and STRO-1-/AP+ subpopulations, recently it has been proposed that the levels of expression of these cell surface antigens provide an indication of osteogenic differentiation in cultures of trabecular explants[243] and BMSC[244] (fig. 4.8). Treatment with TGF-β alone did not alter the proportion of AP+ colonies, irrespective of the timing of its addition. When added in combination with FGF, however, the proportion of AP+ colonies was consistently less than that observed in the corresponding control group.

Treatment with FGF increased the proportion of cells expressing the STRO-1 antigen (fig. 4.6). In the experiment shown, the predominant effect was on the size of the STRO-1+/AP+ subpopulation, but in general, we have also found FGF to increase the size of the STR0-1+/AP- subpopulation[245]. For this effect to be apparent, FGF had to be added early (days 0-7), but not necessarily continuously (compare Ctl with FGF-Ctl and FGF). Treatment with TGF-β alone did not alter the size of STRO-1+/AP- subpopulation but did decrease the size of the dual-labelled (STRO-1+/AP+) subpopulation of cells. When added in combination, TGF-β tended to antagonise the positive effect of FGF on the expression of STRO-1. For both the STRO-1+/AP- and STRO-1+/AP+ subpopulations this is most evident when the effect of continuous treatment with both factors in combination is compared with that of FGF alone.

4.3.5: Colony Area.

Significant increases in mean and total colony area were associated with early treatments of FGF (fig. 4.7). TGF treatments did not significantly affect colony area.

4.5: Conclusions.

FGF is a mitogen for human marrow stromal cells and positively influences their osteogenic differentiation when assessed on the basis of AP expression [245].

Staining cultured human marrow stromal cells with the monoclonal antibodies STRO-1 and B4-78 (anti-bone/liver/kidney isoform AP) reveals the presence of 4 subpopulations of cells that represent cells of the osteogenic lineage at different stages of differentiation (fig.4.8) [243,244]. Treatment with FGF, in addition to increasing total cell number, *selectively* increases the proportion of cells present in the multipotential precursor (STRO-1+/AP-) and committed osteoprogenitor (STRO-1+/AP+) subpopulations. Other potent mitogens in this cell culture system, for example PDGF[230] do not exhibit this same selectivity and do not alter the proportion of cells present in the 4 subpopulations that we have identified.

In this adult human cell culture system TGF-β did not act as a mitogen and, in contrast to FGF, decreased the expression of STRO-1 and the osteogenic marker AP. The limited effect of TGFβ on the expression of STRO-1 is, in our experience, atypical. In general we have found that this factor is a potent inhibitor of STRO-1 expression in this cell culture system[246]. In related studies similar results have been obtained using the TGF-β3 isoform[246]. When added early (days 0 - 7), however, TGF-β significantly increased total colony number. This suggests that TGF-β, possibly through the modulation of matrix synthesis and/or integrin expression, is increasing the proportion of adherent cells in the initial marrow isolate. By decreasing the duration of exposure to TGF-β to 48 hrs and by following this treatment by incubation in the continuous presence of FGF, it may be possible to obtain a large increase in the size of the osteoprogenitor subpopulation in this cell culture system. This could enhance future studies on osteogenic cells *in vitro*. Future studies should address this possibility as well as determining the effects of a brief exposure to TGF-β on the integrins and their extracellular matrix ligands.

Chapter 5: The *In Vitro* Effects Of Parathyroid Hormone Treatments On Bone Marrow Stromal Cells.

5.1: Introduction.

Parathyroid hormone is a major regulator of bone turnover in the adult skeleton. This 84AA polypeptide acts in concert with calcitonin and 1,25 dihydroxy-vitamin D3 to regulate calcium homeostasis. PTH regulates minor fluctuations in extracellular calcium levels primarily by inhibiting calcium excretion from the kidney and by modulating the activity of 25-hydroxyvitamin-D1-hydroxylase which catalyses the formation of 1,25 di-hydroxy-vitamin D3 from 25,hydroxy-cholecalciferol[247,248]. (See fig. 1.14).

PTH exerts its effect on bone metabolism as a consequence of its function to protect against major reductions in extracellular calcium. At times of reduced calcium levels, hypersecretion of PTH from the parathyroid gland causes increased bone resorption and release of calcium to the extracellular serum[248,249]. Although PTH elicits marked increases in bone resorption, conventional wisdom does not consider this to be a consequence of a direct action on osteoclasts. Osteoclasts do not have receptors for PTH[250] and it has been proposed that the actions of PTH on osteoclasts are mediated via osteoblasts[116]or stromal cells[117,118]. It is believed that the bioactivity of PTH resides within the NH_2 terminal residues 1-34 [137,247], epitopes within this region interact directly with a receptor on osteoblasts, affecting proliferation, differentiation and activity of the target cell [251-253].

Interest has focused on PTH since it was demonstrated that PTH could exert anabolic effects on bone[137]. The paradoxical effect of PTH on bone metabolism is not well understood and it has been suggested that different epitope binding sites in the PTH molecule are responsible for the disparate response to the hormone (1.3.1.).
The contradictory effects on bone turnover have also been associated with the temporal administration of the hormone[127, 254-256]. It has also been suggested that the increased bone formation associated with intermittent PTH administration is due to the stimulation of proliferation and differentiation of osteoprogenitor cells in the bone marrow[126].

The effects of PTH on osteoblastic differentiation and bone formation have been studied both *in vivo* and *in vitro*. The *in vivo* anabolic effects of intermittent PTH administration on experimental animals[140,257,258] and human subjects [137,138] are clearly demonstrated. The *in vitro* effects of PTH administration on osteoblast differentiation however, remain contradictory, controversial and differ depending on the model used and the cell's differentiation status[259-265]. Because of the significant differences seen between the different culture systems and because differences are

apparent between human and experimental animal models it was considered essential to use cells derived from human bone marrow cells for this investigation, which examines the temporal effects of treatments with hPTH(1-34) on parameters of cell growth and differentiation *in vitro.* In addition the interaction between PTH and dexamethasone (Dx), a synthetic derivative of the glucocorticoid cortisol, on the same parameters was investigated. Glucocorticoids are known to increase the proliferation and/or osteogenic differentiation of marrow stromal cells derived from certain species including rats and humans[178,181,266-268]. Importantly, Dx is reputed to augment the ability of marrow stromal cells to respond to PTH[179]possibly by enhancing ligand binding to the receptor or increasing PTH receptor expression[269].

A pilot experiment was undertaken to assess the dose response relationship of PTH treatment on the proliferation and osteogenic differentiation of bone marrow stromal cells. Cell morphology, cell numbers, surface expression of STRO-1 and AP, CFE and colonies expressing AP, an early indicator of osteogenic differentiation in marrow stromal cultures[180], were assessed. The anabolic effect of PTH is thought to be mediated via the cAMP/PKC pathway[109,125,142,144,161,270], and as this response is accepted as a characteristic of the osteogenic phenotype[271], cAMP response to the PTH treatments was also measured.

5.2: Methods.

Bone marrow cells were obtained as described previously, from segments of rib removed from a female patient aged 68, during routine thoracic surgery at Frenchay Hospital, Bristol. Cells were plated at a density of 2 x $10^5/cm^2$ in standard media formulation plus 100μM ascorbate-2-phosphate supplemented with hPTH(1-34) at 0.1nM, 1.0nM, 10nM and 100nM, in the presence and absence of Dx. To assess the effects of PTH on cell numbers and for analysis of surface STRO-1 and AP expression by flow cytometry, cells were seeded in T75 culture flasks (x4/treatment/analysis) and cultured for 21 days. To measure intracellular cAMP levels, cells were plated in 6 well plates (x1 plate/treatment), and to estimate the effects on colony forming efficiency (CFE), and colony area, cells were seeded in petri dishes (4 petris/treatment) and cultured for 21 days.

Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. After 7 days in culture, the non-adherent cells were removed then replenished with the appropriate medium twice weekly thereafter. At the appropriate time point, the cells were harvested and parameters assessed according to methods described in Chapter 2.

Briefly, cells were counted on a Coulter counter electronic cell counter. Colonies were fixed and stained with methylene blue to evaluate the total number and Fast Red TR to estimate the number expressing AP. cAMP values were calculated according to instructions supplied with the Biotrak cAMP enzymeimmunoassay system and normalised to fMol cAMP/mg protein using the Bio-Rad DC protein assay. Flow cytometry was carried out according to the methods of Simmons[208]. Image analysis (NIH Image v1.61) was used to manually measure the area of the colonies.

All experiments were repeated at least once and data from a single representative experiment is shown. The distribution of the data was confirmed as normal using the Shapiro Wilk W test, where data was not normally distributed, a log transformation was used to normalise the distribution prior to test. Differences in the data were assessed using the one way Analysis of Variance followed by the Tukey-Kramer test of Honest Significant Difference ($p < 0.05$).

5.3: Results.

5.3.1: Cell Morphology.

The presence of PTH at concentrations of 10nM and 100nM affected cell morphology. In control cultures, cells were fibroblastic in shape (fig. 5.1a) This differed from the more elongated fibroblastic as well as the cuboidal morphology seen in the PTH treated cultures (fig 5.1c) Treatment with Dx resulted in a morphological transformation of the cells from fibroblastic (fig 5.1a) to elongated fibroblastic (fig. 5.1b). Treatment with PTH and Dx resulted in cultures with elongated fibroblastic and cuboidal morphology (fig. 5.1d) similar to that seen in the PTH treatments but with an apparent increase in density.

5.3.2: cAMP Response.

Treatment with PTH ≥ 10nM significantly increased the intracellular cAMP levels compared to the control, (fig. 5.2.) Cultures treated with 0.1nM PTH had similar levels of intracellular cAMP as the control, 1.0nM treated cells had a small insignificant reduction in cAMP concentration.

In cultures where PTH was added in combination with Dx, treatment with PTH at all doses tended to increase the levels of intracellular cAMP. Only at doses ≥ 10nM however, did the increase attain statistical significance.

5.3.3: Cell Numbers.

Treatment with 100nM PTH increased cell number significantly (fig. 5.3), irrespective of the absence or presence of Dx. The combined effect of 100nM PTH + Dx was greater than that of either factor alone ($p < 0.05$).

5.3.4: Total Colony Numbers.

Treatment with PTH at 10nM increased the colony forming efficiency of marrow cell suspensions ($p < 0.05$) (fig. 5.4a; 5.5a).

Treatment with Dx alone tended to increase the colony forming efficiency of marrow cell suspensions (fig.5.4b; 5.5a). When added in combination with 100nM PTH the number of colonies formed was greater ($p < 0.05$) than that treated with either factor alone.

5.3.5: Colony Area.

Mean area

The mean area of the colonies formed in cultures treated with PTH or Dx was similar to that of the control cultures. This same parameter was increased significantly however in cultures treated with 100nM PTH and Dx in combination (fig.5.6a)

Total area.

Using a single representative plate from each treatment group, the total colony area was found to be notably increased in the 10nM treatment compared to the control, when PTH was administered alone (fig. 5.6b).

In cultures where PTH was combined with Dx, a considerable increase in total area was seen in the 100nM Dx cultures, ≈ x4 compared to control (fig. 5.6b).

§12345

5.3.6: AP+ Colonies.

Treatment with PTH or Dx did not alter the number of AP + colonies (figs. 5.4c,d; 5.5b).

Treatment with Dx and 100nM PTH in combination however induced a significant increase in the number of AP+ colonies.

5.3.7: AP+ Colony Area.

Mean area.

PTH treatment was related to insignificant increases in mean colony area (fig. 5.7a). whereas treatment with PTH+Dx had no general effect.

Total area.

Cultures treated with PTH had greater total colony areas than the control although the differences between treatments was comparatively small (fig. 5.7b).

The Dx supplemented cultures showed similar colony areas except in the case of the 100nM Dx treatment which was ≈ x 20 greater than the next highest treatment (fig. 5.7b and fig. 5.4c,d).

5.3.8: Percentage of Colonies Expressing AP.

In the presence of PTH, Dx or both factors in combination, there was a general trend for the percentage of AP+ colonies formed to be increased (fig. 5.8a). In every case however, the observed increase did not attain statistical significance

The number of AP+ colonies strongly correlated with the total number of colonies, adj. $r^2 = 0.82$, ($p < 0.001$) (fig. 5.8b)

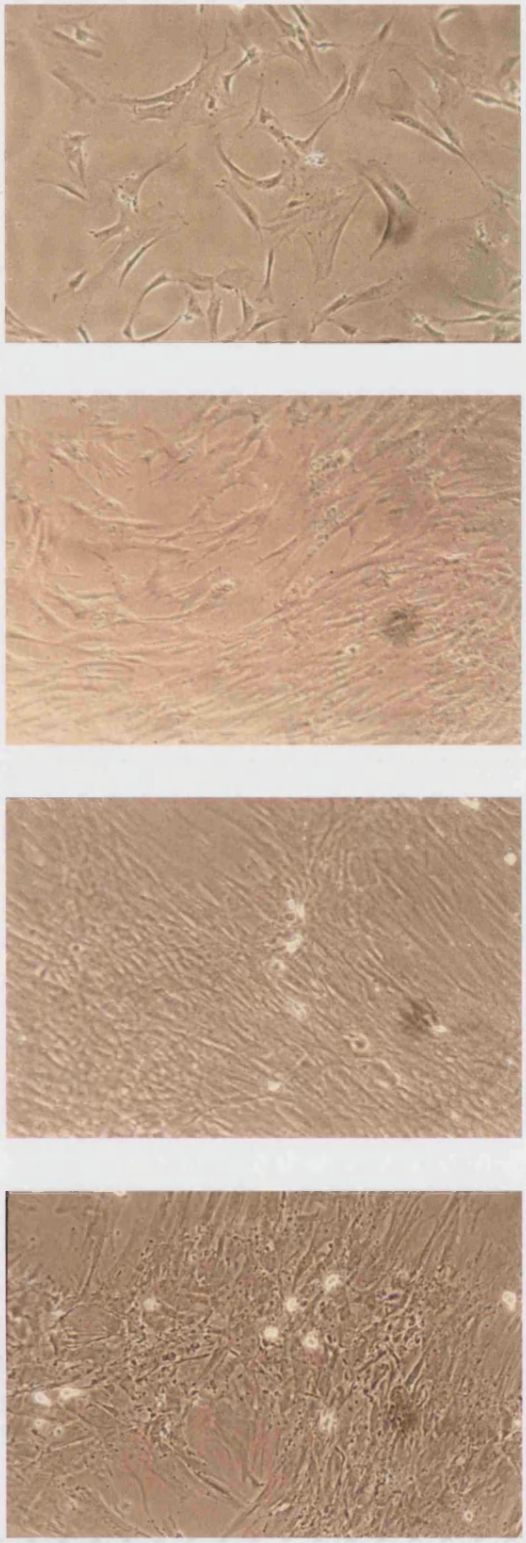
5.3.9: Flow Cytometry.

In cultures treated with PTH only, the sub-population distribution in the 0.1nM, 1.0nM and 10nM treatments did not differ dramatically from the control (fig. 5.9b-e; simplified in fig. 5.11a). In the 100nM treated cultures (fig. 5.9f; 5.11a), only 15% of cells were present in the -/- population suggesting a shift in the cell distribution towards the sub-populations associated with the osteogenic pathway.

In the PTH/Dx treated cultures, the sub-population distributions in the 0.1nM Dx and 1.0nM Dx cultures were similar to the control (5.10b-d; 5.11b) These PTH/Dx treated groups differed from the control only in that they had a modest shift in the sub-population distribution from the -/- towards the osteogenic phenotype. The 10nM Dx treatment differed noticeably from the control with marked increases in the STRO-1+/AP-, and STRO-1+/AP+ sub-population (5.10e; 5.11b). Additionally, the STRO-1-/AP+ population in the 10nM Dx cultures was much less than the control value. The 100nM Dx cultures when compared to the control revealed a large shift in the sub-populations towards the osteogenic phenotype (5.10f; 5.11b).

Figure 5.1 Photomicrographs (x 400 orig. mag.) at 21 days culture.
(a) Control; (b) Control Dx; (c) PTH (10nM); (d) PTH (10nM) Dx

Figure 5. 1 (a-d): Effect of PTH and Dx Treatments on Cell Morphology.



(a)

(b)

(c)

(d)

Figure 5. 2: Effect of PTH Treatments on Intra-Cellular Levels of cAMP.

Error bars represent S.E. of mean; n = 6;

* $p < 0.05$ compared to relative control

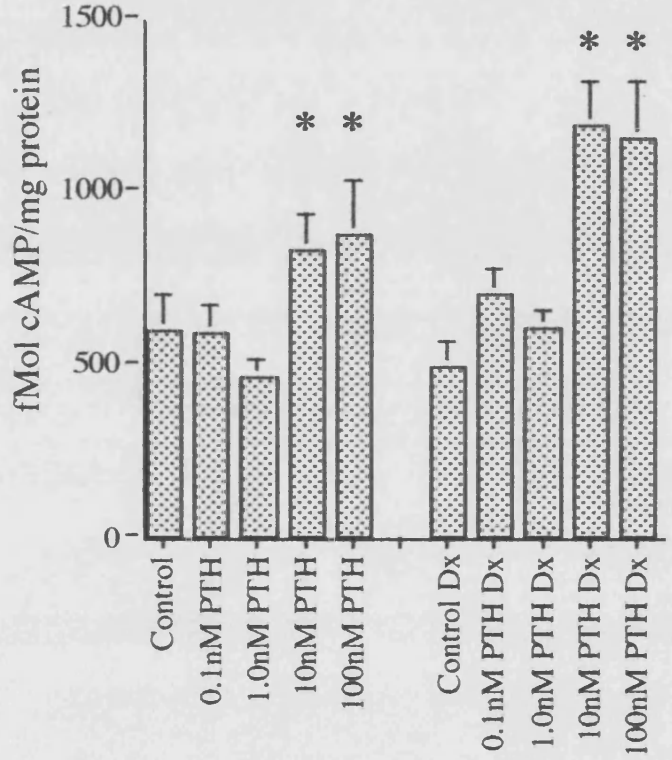


Figure 5. 3: Effect of PTH±Dx Treatment on Cell Numbers.

Error bars represent S.E. of mean; n = 4;

* $p < 0.05$ compared to relative control

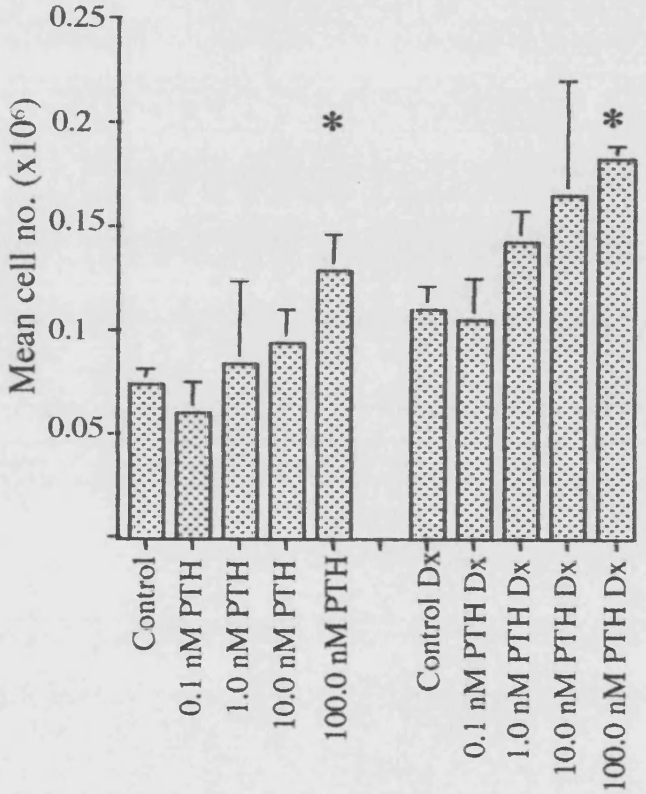
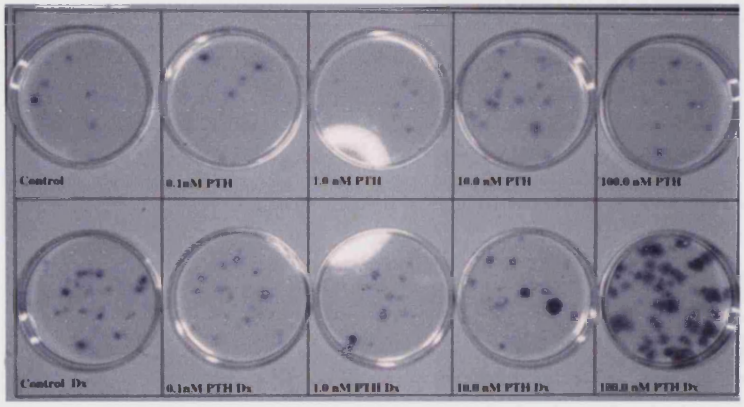


Figure 5.4: Photographs showing colony coverage at 21 days
in response to (a) PTH; (b) PTH Dx.
AP+ colony coverage at 21 days
in response to (c) PTH; (d) PTH Dx.

Figure 5. 4 (a-d:) Effect of PTH Treatment on Colony Numbers and AP+ Colony Numbers.

Control 0.1nM 1.0nM 10nM 100nM

-Dx



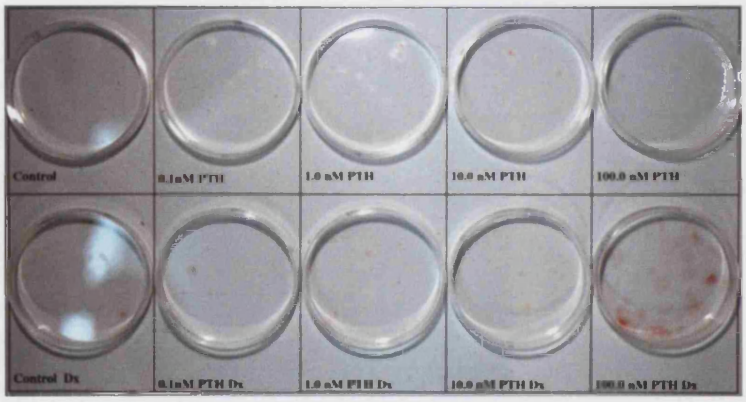
(a)

+Dx

(b)

Control 0.1nM 1.0nM 10nM 100nM

-Dx



(c)

+Dx

(d)

Figure 5. 5: Effect of PTH Treatment on (a) Colony Number and (b) AP Colony Number.

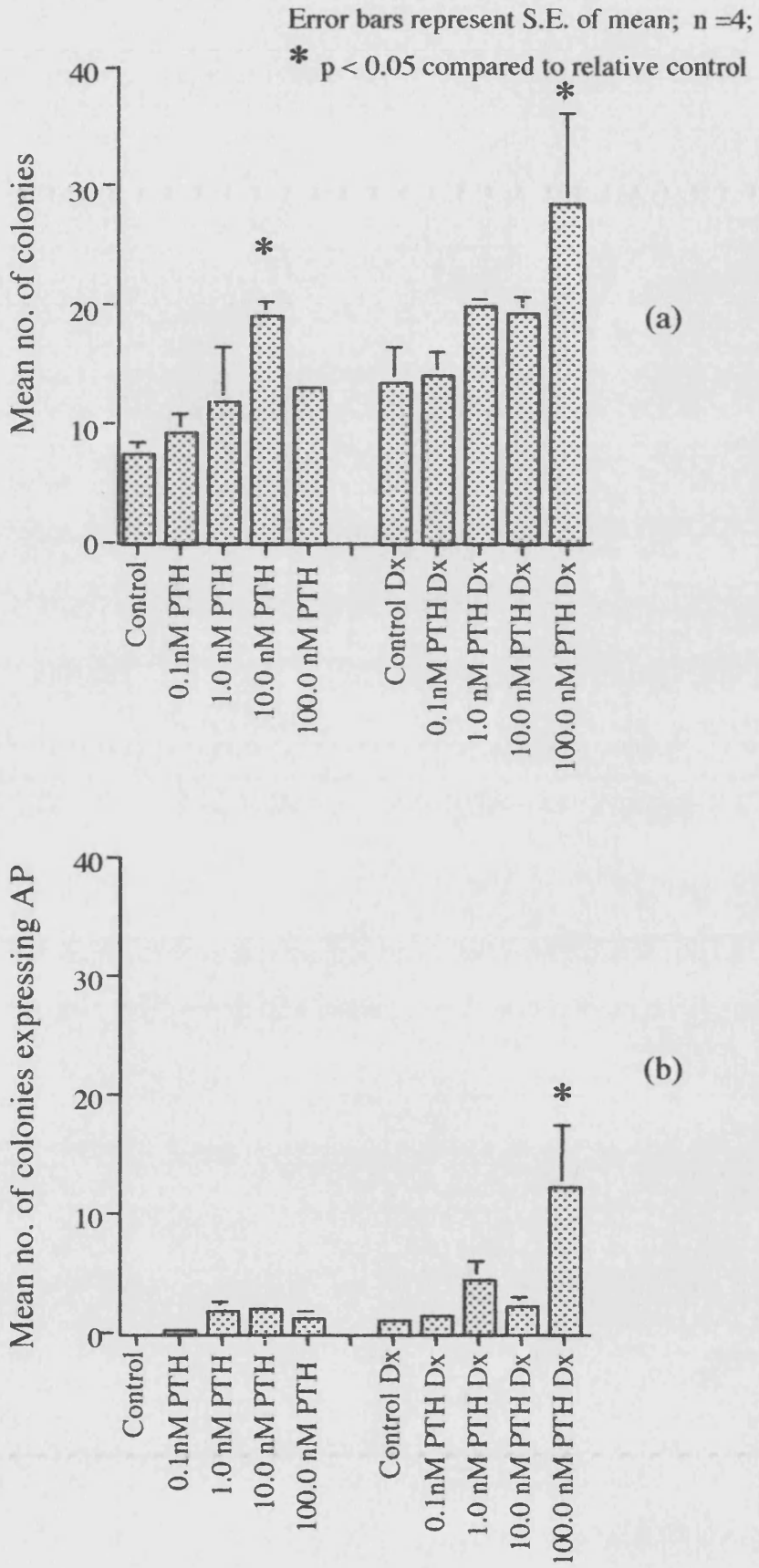


Figure 5. 6: Effect of PTH Treatment on (a) Mean Colony Area, and (b) Total Colony Area

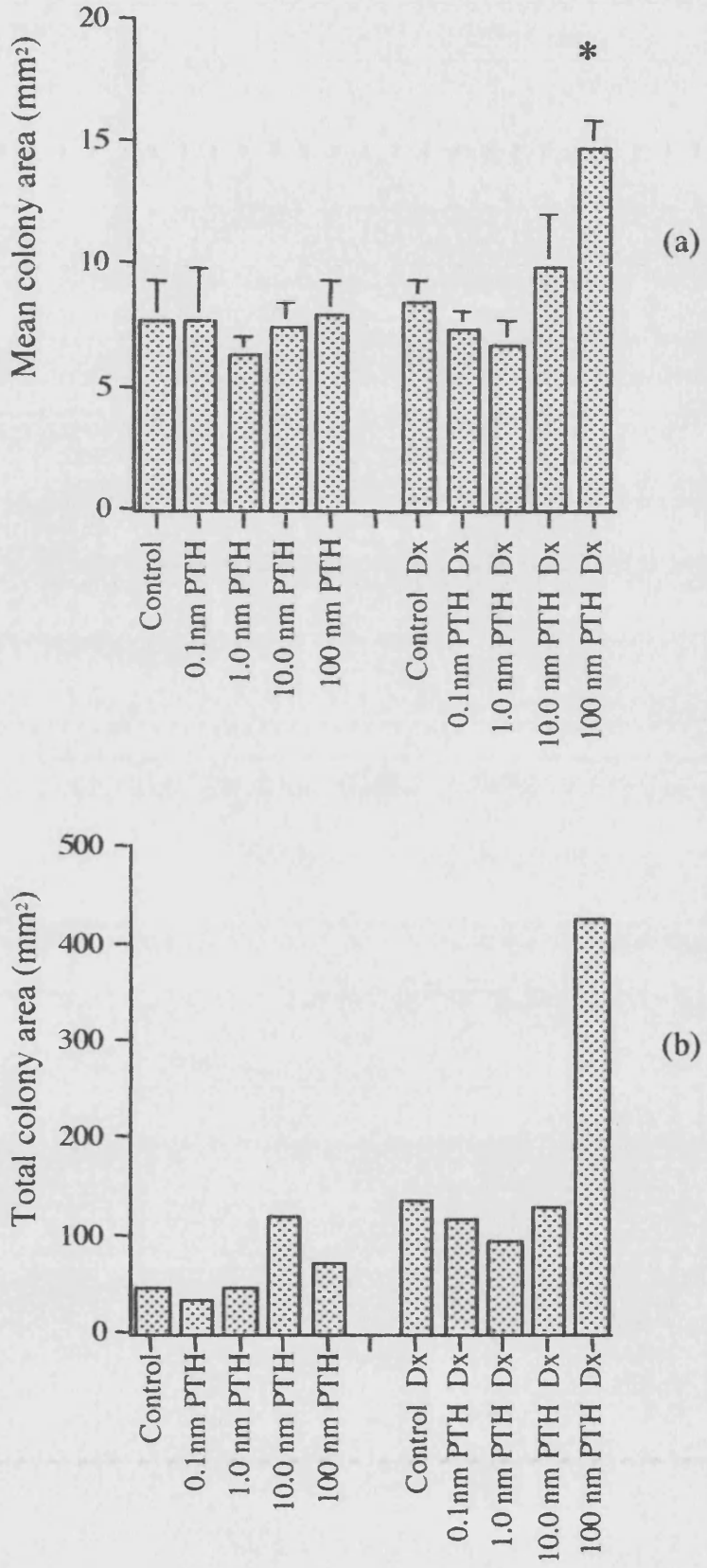


Figure 5. 7: Effect of PTH Treatment on (a) Mean AP+ Colony Area, and (b) Total AP+ Colony Area.

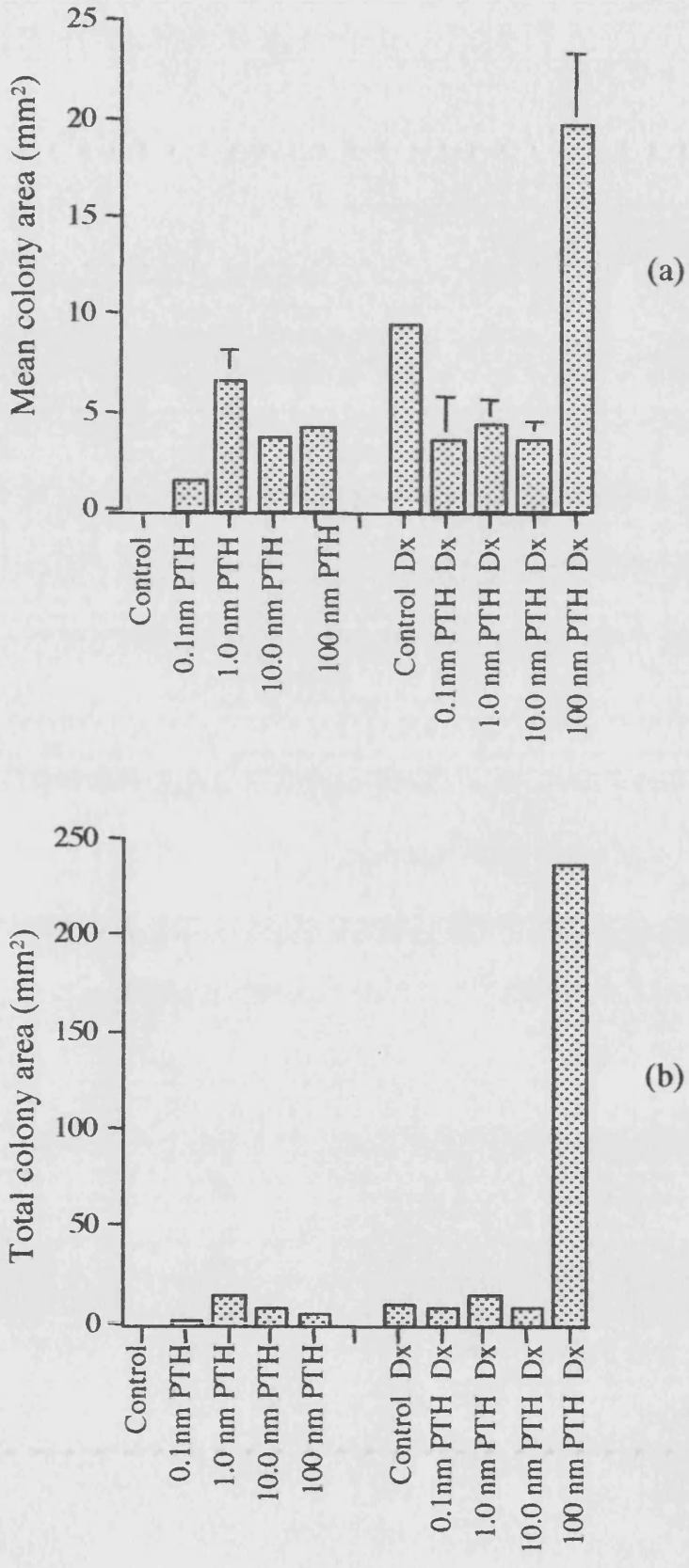
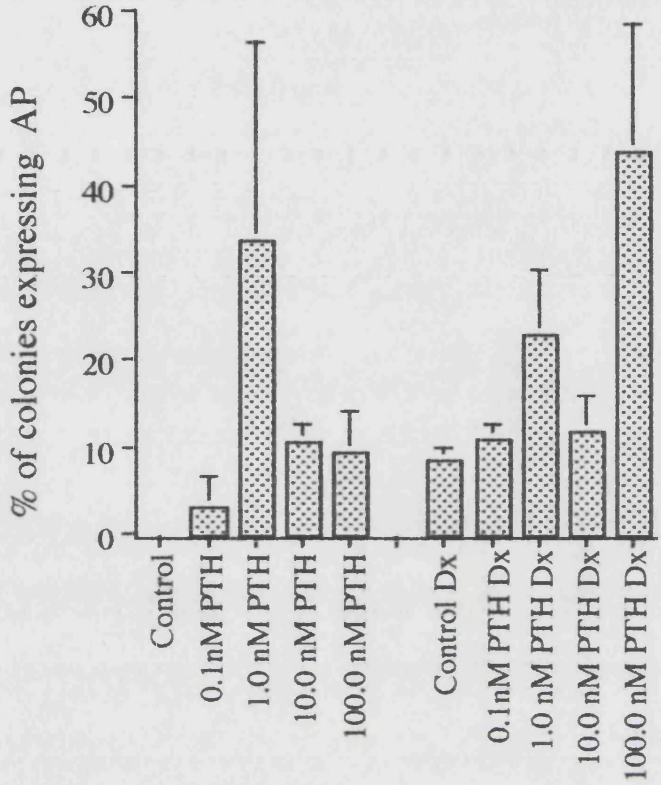
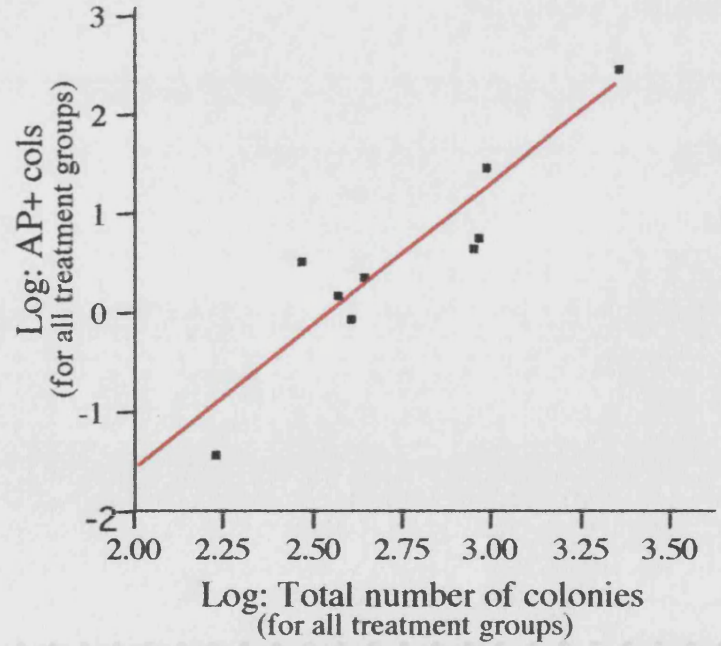


Figure 5. 8: (a) Effect of PTH Treatment on the % of Colonies Expressing AP;
(b) Correlation between the Number of AP colonies and Total Number of Colonies.



(a)

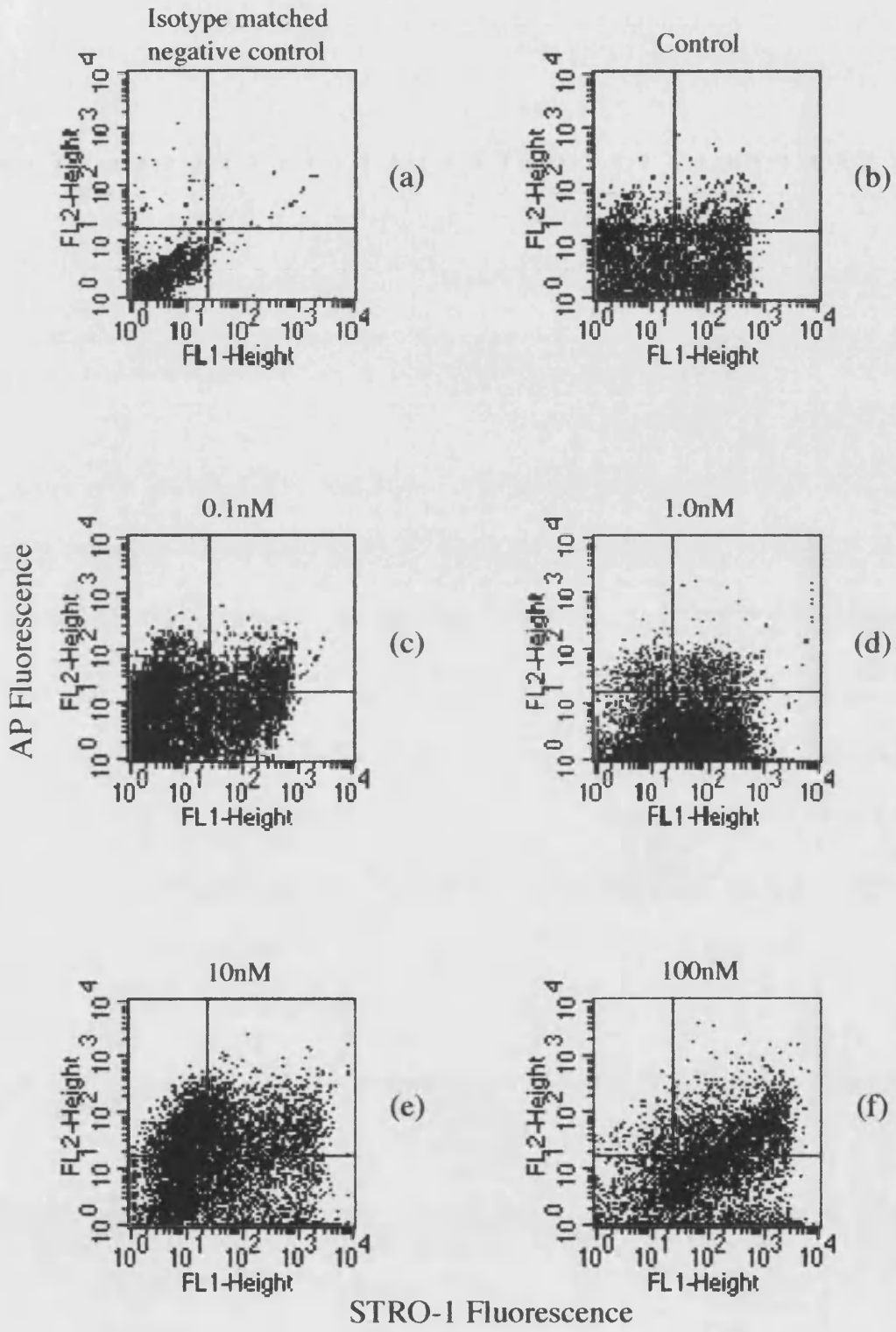


(b)

Adj.$r^2 = 0.82$

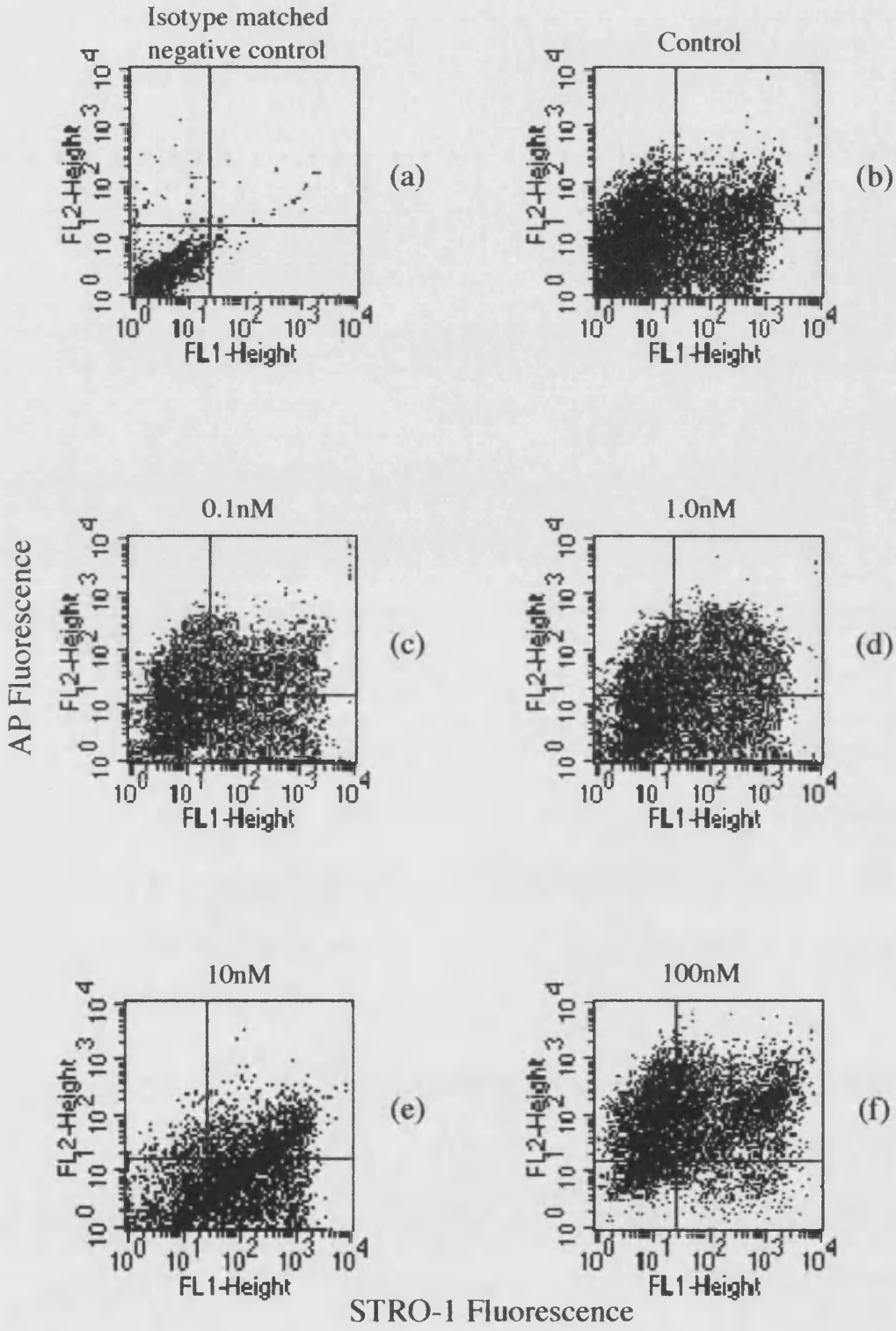
$P < 0.001$

Figure 5. 9 (a-f): Effect of PTH Treatment on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.



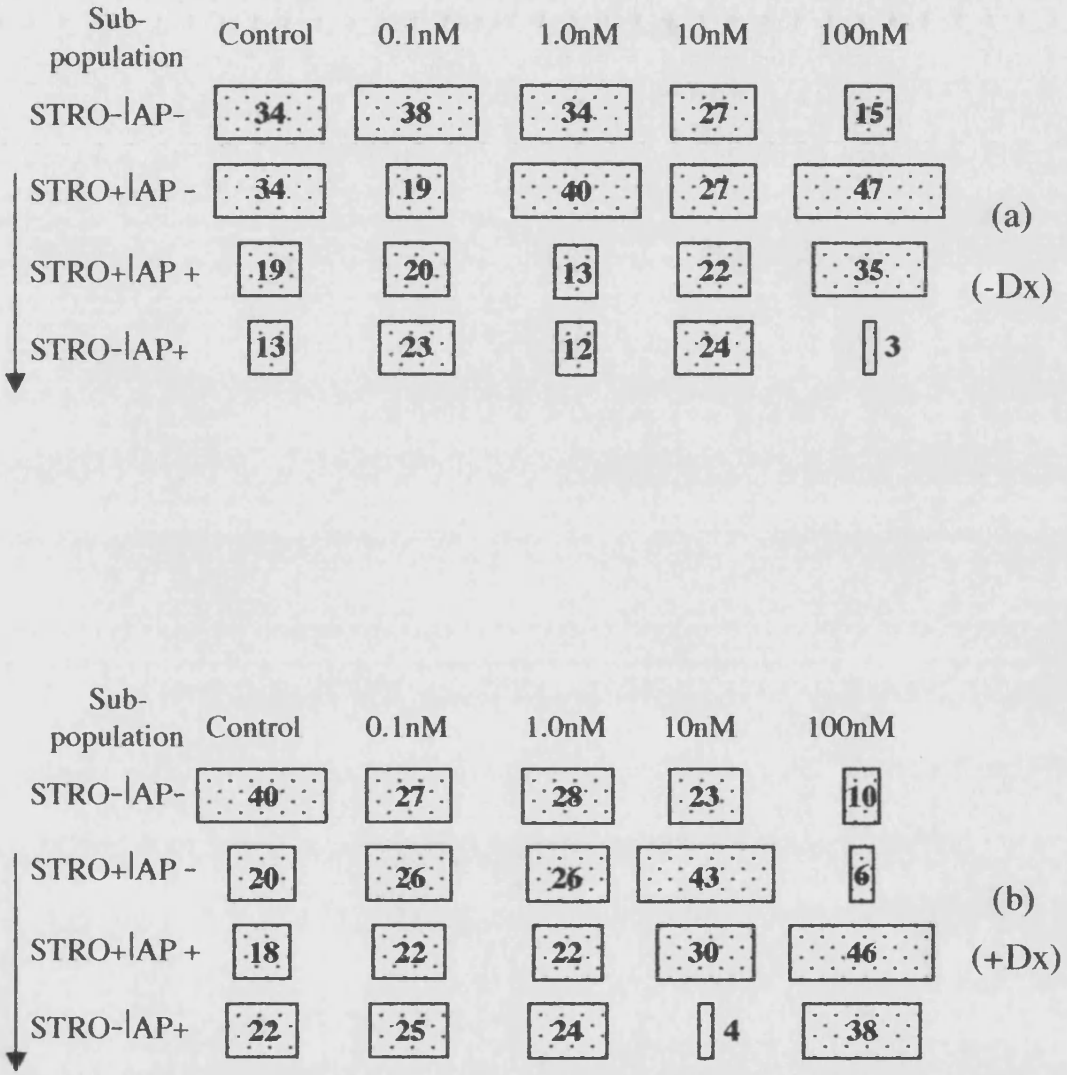
Cells that had been dual-labelled for expression of STRO-1 and AP antigens were visualised as dot plots with green fluorescence (FITC) on the *x* -axis and red fluorescence(R-PE) on the *y*--axis. Quadrants were set using the negative control sample such that >95% of cells appeared within the lower left quadrant (a).

Figure 5. 10 (a-f): Effect of PTH Dx Treatment on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.



Cells that had been dual-labelled for expression of STRO-1 and AP antigens were visualised as dot plots with green fluorescence (FITC) on the *x* -axis and red fluorescence(R-PE) on the *y*--axis. Quadrants were set using the negative control sample such that >95% of cells appeared within the lower left quadrant (a).

Figure 5. 11: Effect of PTH±Dx Treatment on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.



Relative proportions of sub-populations of cells labelled with the STRO-1 and AP antibodies. Block areas represent relative sub-population sizes, numbers associated with blocks are percentage of cells (to nearest percent). Arrows represent the direction of increasing osteogenic differentiation.

5.4: Discussion.

Treatment with hPTH (1-34) in the range 10-100nM caused early changes in bone marrow stromal cell (BMSC) morphology. Previous studies have demonstrated that treatment with PTH (1-34) or its analogues, produced morphological changes in osteoblast-like cells[272,273] and osteoblasts, both in vitro[274] and in vivo[16]. These studies described changes at an ultrastructural level, in the nuclear matrix and cytoplasm, so it seems unlikely that changes of this magnitude would be visible at the magnification shown. Furthermore, as BMSC cultures are heterogeneous, with osteoblasts and their precursors representing a minority constituent at this stage of culture[178,275], such morphological changes would therefore not lead to the overall change in morphology seen in these groups.

PTH treatments have been shown to increase proliferation in osteoblast-like cell lines[252,276], trabecular bone-derived cells of human[259] and rat origin[144,277] as well as increase the proliferation of osteoprogenitor cells in cultures of rat bone marrow[126]. It therefore seems feasible that the change in cell morphology associated with PTH treatments could be related to an increase in cell density caused by increased proliferation. However, the PTH associated changes in cell numbers seen this experiment were not dramatic (maximum of 1.75 and 1.66 fold increase with PTH±Dx) and therefore makes this an unlikely explanation.

Inter-treatment variations in morphology could be due to a disparity in differentiation stage, PTH has been shown to stimulate osteogenic differentiation in trabecular explants[120], ex vivo marrow cultures[126] and in osteoblast-like cells[260] and it follows that the PTH treated cultures may have advanced further along the differentiation pathway than the untreated controls, and reflect this maturation with a change in morphology. However, given that the osteogenic influence of PTH is directed against the osteogenic sub-population and that this population is relatively small, then one would not expect to see the global effect on cell morphology that was seen in these cultures

cAMP has been shown to alter the morphology in a wide range of cell types *in vitro* [278-281] and in this system an intracellular cAMP response to PTH treatments may be responsible for differences in cell morphology. This concept is supported in part, by the observation that the changes in cell morphology among the PTH treatments were only seen in the 10nM and 100nM cultures, and these were the only groups which showed an increase in intra-cellular cAMP in response to hPTH. When cells were treated with hPTH accompanied with Dx, the greatest change in morphology was

seen in the 10nM±Dx and 100nM±Dx treatments and these were the only groups where the cAMP response to hPTH was significantly higher than the control. The importance of this change in cell morphology is underlined by work which demonstrates that PTH alteration of osteoblast membrane, cytoskeletal and nucleoskeletal proteins may direct PTH signalling pathways to target genes and modulate osteoblast gene expression[274].

Treatment with hPTH at 100nM caused a significant increase in cell numbers. A similar pattern was seen in cultures which were additionally supplemented with Dx with the exception that the increase at both the 10nM and 100nM doses reached significance. A number of mechanisms could be responsible for this increase in cell numbers. PTH has been previously shown to increase cell proliferation in a variety of osteogenic *in vitro* systems[126,144,252,259,276,277,282]. A number of studies suggest that the PTH associated increase is mediated via the cAMP/PKA pathway [144,277,283-285] but the overall evidence is contradictory, with others reporting that PTH stimulates proliferation by a mechanism not using the cAMP/PKA pathway [286,287]. An examination of the relationship between intra-cellular concentrations of cAMP and cell numbers in this study reveals a correlation of adj. $r^2 = 0.38$; $p = 0.03$. (least squares regression analysis, Anova; (not shown)). This equates to a moderate but statistically significant association between intracellular cAMP levels and cell numbers and therefore suggests that other pathways may act in association with cAMP/PKA to bring about change. This concept is supported by reports that the cAMP/PKA pathway acts in concert with the PKC paths to effect increases in proliferation [144].

Recent reports suggest that the increase in cell numbers associated with PTH treatment is not due to an increase in proliferation but caused by a reduction in osteoblast apoptosis[288]. It is proposed that PTH acts directly on osteoblasts and initiates the cAMP/PKA pathway which then interferes with some death pathways. As mentioned above, the level of association seen in this study between cell numbers and intracellular cAMP suggests the co-involvement of other signal pathways in the PTH associated increase in cell numbers

An increase in cell numbers would also result from any mechanism which enhanced the capability of the normally non-adherent BMSC population to convert to the adherent cell type. It has been proposed that these non-adherent cells represent a differentiation stage which precedes that of the adherent cells[289]. There is evidence that some bone anabolic agents, which include PTH, enhance both bone cell numbers and bone formation by recruiting osteoblast precursors present in the non-adherent

population and promoting them to the more differentiated adherent population[289-291]. The involvement of cAMP is also implicated in this process,[290] so, it can be seen that activation of the cAMP/PKA pathway is an important component in the anabolic response associated with PTH administration.

The evidence provided by this experiment, and that of work already discussed, is not sufficient to define a precise mechanism by which PTH treated cultures have increased cell numbers. It is likely that increased cell proliferation, cell adherence and a reduction in apoptosis all act in concert to bring about a net gain, but an elucidation of this effect will require the study of other parameters.

Treatment with hPTH in the 0.1nM-100nM±Dx range increased the numbers of colonies compared to the control, and the increase was statistically significant in cultures treated with 10nM and 100nM Dx. Comparing the ±Dx treatments revealed a trend where the application of Dx increased the CFE. This finding is generally in accordance with earlier work which demonstrated that the administration of Dx to human culture systems increases the proportion of cells with osteogenic potential [292,293] and upregulates colony forming efficiency[180]. The reason for the Dx associated increase in CFE is largely unknown but is thought to be linked to its effect on primary cell adhesion [180].

Previous work on the effects of PTH on colony forming efficiency in cells derived from mouse marrow revealed no difference in cultures treated with PTH[294]. This was in contrast to the findings from a rat model where PTH increased colony forming efficiency by 25%[289]. In an ex-vivo model, cultures of marrow explants from rats treated with PTH yielded a greater number of colonies (320%) than the vehicle treated controls[126].

In this study, the increase in the number of colonies in cultures treated with PTH could be due to an overall increase in cell numbers. Plotting cell numbers against colony number, least squares regression analysis reveals a correlation coefficient of adj.r^2 = 0.69 (p = 0.002). This suggests that the number of cells in a culture is an important and highly significant factor which influences the process of colony formation. This could simply be because any increase in the number of cells will concomitantly lead to an increase in the number of cells which have the ability to form colonies. If the increased cell numbers are due to a PTH induced increase in proliferation then it follows that colonies in the PTH treated cultures would be larger. An examination of mean colony areas in groups where 0-100nM PTH was applied,

revealed no real inter-treatment differences. The overall colony coverage per treatment group was a function of the number of colonies (adj.r^2 = 0.80; p = 0.02) rather than the mean area of the colonies (adj.r^2 = -0.31; p = 0.86). This suggests that in these groups, the overall increase in cell numbers is due to a mechanism other than increased proliferation. As discussed above, agonists which increase intracellular levels of cAMP have been shown to induce the transition of the normally non-adherent BMSC to become adherent[289-291] thereby increasing the number of cells in culture. In this study, the increase in the number of cells and colonies in PTH treated groups could be explained by this model.

Where PTH was applied with Dx, no real difference was seen in mean colony areas in the 0.1-10nM PTH Dx range. A significant increase in area was seen in the 100nM Dx cultures. The overall colony coverage per treatment group was more closely related to the mean area of the colonies (adj.r^2 = 0.86* ; p = 0.01) rather than the total number of colonies (adj.r^2 = 0.34*; p = 0.17) suggesting the influence of proliferation on cell numbers was greater in cultures supplemented with Dx. On closer analysis it can be seen that this is probably not the case. Only in the 100nM Dx cultures was there any evidence of greater proliferation in the form of increased mean colony area, it was however of sufficient magnitude to skew the result in that direction.

In cultures further supplemented with Dx the increase in colony number in the range 0.1-10nM PTH is likely due to the transition of the normally non-adherent BMSC to adherent and, in the 100nM Dx group it is likely that this mechanism is augmented by a concomitant increase in proliferation.

Expression of AP is an early and widely accepted sign that a cell has initiated a program of osteogenic differentiation[295]. The number of colonies expressing AP was increased in cultures treated with PTH±Dx and this increase is consistent with an enhancement of osteogenic differentiation[180]. However, this increase only reached significance in the presence of 100nM PTH+Dx. The percentage of total colonies which expressed AP varied widely between the treatment groups although none of these differences were significant. This could be due to the small numbers of AP+ colonies present. The number of AP+ colonies per treatment group was closely correlated of the total number of colonies (adj.r^2 = 0.76; p <0.001) and this information along with the percentage data provides evidence that the increase in AP+ colonies and osteogenic enhancement may not be a direct result of PTH administration *per se* but could be a function of increased colony forming efficiency.

* Plotting the natural log of the data to normalise the distribution.

An estimate of the differentiation status of the cultures was achieved by analysis of the expression of cell surface antigens STRO-1 and AP. Recently it has been proposed that the levels of expression of these cell surface antigens provide an indication of osteogenic differentiation in cultures of trabecular explants[243] and BMSC[244].

In cultures treated with 0.1nM and 1.0nM PTH the percentage of cells in the STRO-1 -/AP- population were similar to that of the control. This group is the most heterogeneous of the 4 sub-types and is thought to contain very early uncommitted cells of the osteoblast lineage, as well as late stage cells - osteocytes/lining cells [244]. This suggests at these doses, PTH does not promote the progression of the early uncommitted cells of the STRO-1 -/AP- population to a more differentiated state. Analysis of the sub-populations representative of the more differentiated phenotypes failed to reveal any obvious pattern associated with PTH treatment. In the 0.1nM treatments, the percentage of cells in the STRO-1/AP+ population, representing the most differentiated sub-type, was almost double that seen in the control group, however, this percentage returned to control levels in the 1.0nM cultures. Considering this data along with the cAMP, cell number and colony data suggests that treatment with PTH at 0.1nM and 1.0nM concentrations has little or no effect in this system.

In the 10nM and 100nM cultures there was a reduction in the proportion of cells in the STRO-1 -/AP- population. This may suggest that these concentrations of PTH promote the early uncommitted cells of the STRO-1 -/AP- population to a more differentiated state. In the 10nM treatment the proportions of cells tended to be evenly spread among the population sub-types. In the 100nM cultures however, a large shift, compared to any of the other groups was seen in the distribution of populations. In this group 85% of cells were present in the osteogenic differentiation pathway but their distribution was strongly skewed towards the less differentiated state. In this group only 3% of cells were present in the STRO-1-/AP+ population. This may indicate that PTH enhances the recruitment of early uncommitted cells to the osteogenic differentiation pathway but inhibits their progress when they reach a certain, as yet unidentified, differentiation stage.

In cultures where PTH was supplemented with Dx, all PTH treatments in the range 0.1-100nM were associated with an increase in the proportion of cells present in the committed pathway. In the 0.1nM and 1.0nM cultures, the distribution of cells among the committed sub-populations was similar to that seen in the control group and this reinforces the earlier supposition that at these concentrations PTH exerts a negligible or zero effect. In the 10nM culture the distribution was very similar to that of the

100nM -Dx. In the 100nM Dx group, 90% of cells were present in the committed pathway and in contrast to the comparable -Dx treatment, the distribution was strongly skewed towards the more differentiated state. In this system increased colony area provided evidence of increased proliferation and that may account for increased proportions of cells with osteogenic potential. The presence of Dx would accelerate their progress along the differentiation pathway.

5.5: Summary.

PTH treatment: PTH at doses ≥ 10nM does not affect parameters of growth and differentiation in this cell culture system. Concentrations of 10nM and 100nM increase cell numbers most likely by enhancing cell adhesion rather than increasing proliferation as evidenced by increased colony numbers but not colony area.
Osteogenic differentiation is not enhanced by 10nM and 100nM PTH as evidenced by the proportion of AP+ colonies and STRO-1, AP cell surface expression. Increased intracellular cAMP is likely to be an important mediator of the actions of PTH.

PTH+Dx treatment: Changes similar to those listed above were noted with the following exceptions; 100nM PTH+Dx synergistically enhanced cell number by increasing proliferation and adhesion (colony areas and numbers increased) and also increased osteogenic differentiation (AP+ colonies and AP+ cells increased). Osteogenic differentiation was increased compared to comparable -Dx cultures. (Increased AP+ colonies probably due to presence of Dx).

If PTH exerts a significant influence on BMSC via its effects on early (uncommitted) cell adhesion/recruitment then the timing of PTH treatment is an important parameter and may effect cell responses to treatment. This concept is supported by evidence that PTH exerts dissimilar effects which are treatment time dependant[160]. Suggestions that the PTH target cell is an immature osteoblast[294] or preosteoblast [296] add further weight to this argument.

To investigate this, subsequent experiments will add a temporal aspect to the administration of PTH with comparisons made between early, late and continuous treatments. Experiments will also be extended to investigate possible mechanisms for the interaction between PTH and dexamethasone on BMSC

Chapter 6: The *In Vitro* Effects Of Delayed Parathyroid Hormone Treatments On Bone Marrow Stromal Cells.

6.1: Introduction.

The PTH dose response experiments demonstrated that PTH at concentrations of 10nM and 100nM are capable of increasing the number of colonies, cells and, when applied with Dx, AP+ colonies in cultures of human BMSC.

On the basis of these findings it was postulated that PTH targets early (uncommitted) cells and affects the adhesion and/or recruitment to the osteogenic lineage in cultures of BMSC. To test this hypothesis, experiments were performed in which PTH was applied from the start of the culture period (continuous), and after 48 hours had elapsed (delayed). In both instances, treatment was then continued until the end of the culture period. The rationale for this approach is, that in the delayed experiment, cells in the non-adherent fraction are removed prior to the application of PTH thereby depleting the cultures of the putative target cell population (pre-CFU-F).

If, as has been previously suggested however, the target cells are the immature osteoblast and/or preosteoblast[294,296] then the delayed treatment will bypass the very early differentiation stages of these cell types, this delayed addition of PTH will have effects on parameters of growth and differentiation distinguishable from those observed in the continuous group.

6.2: Materials and Methods.

Bone marrow cells were obtained as described previously, from segments of rib removed from a patient aged 31 of unknown sex during routine thoracic surgery at Frenchay Hospital, Bristol. Cells were plated at a density of 2 x $10^5/cm^2$ in standard media formulation plus 100μM ascorbate-2-phosphate supplemented with hPTH(1-34) at 10nM and 100nM, in the presence and absence of Dx.

To assess the effects of PTH on cell numbers and for analysis of the surface expression of STRO-1 and AP by flow cytometry, cells were seeded in T75 culture flasks (x4/treatment/analysis) and cultured for 21 days. To estimate the effects on colony forming efficiency (CFE), colony area and optical density, cells were seeded in petri dishes (4 petris/treatment) and cultured for 21 days.

Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. After 48 hours in culture, the non-adherent cells were removed, cultures were replenished with appropriate medium and fed twice weekly thereafter. At the appropriate time point,

the cells were harvested and parameters assessed according to methods described in Chapter 2.

All experiments were repeated at least once and data from a single representative experiment is shown. The distribution of the data was confirmed as normal using the Shapiro Wilk W test, where data was not normally distributed, a log transformation was used to normalise the distribution prior to test. Differences in the data were assessed using the one way Analysis of Variance followed by the Tukey-Kramer test of Honest Significant Difference ($p < 0.05$).

6.3: Results.

6.3.1: Cell Morphology.

The continuous presence of PTH at concentrations of 10nM and 100nM affected the morphology of the cells in a similar manner to that seen in PTH experiment 1 (not shown). Where PTH was applied late, no obvious change was seen in cell morphology. Dx produced changes as previously seen in PTH experiment 1.

6.3.2: Cell Numbers.

Where PTH was applied without Dx, no effect was seen with either continuous or delayed treatments (fig. 6.1a,b). The addition of Dx to the system caused a significant increase of cell numbers in the continuously treated 100nM cultures and a significant decrease in the delayed treated 10nM cultures ($p < 0.05$).

Comparing the continuous to the late treatments, the number of cells was less in all the late cultures. This reduction was significant in the 100nM, 10nM Dx and the 100nM Dx cultures ($p < 0.05$).

6.3.3: Total Number of Colonies.

Continuous treatment with PTH alone increased the total number of colonies, and this increase was significant in both the 10nM and 100nM cultures (figs. 6.2a; 6.3a).
Cultures treated with PTH and Dx showed a significant decrease in the number of colonies in the 10nM Dx treatments and a small insignificant increase in the 100nM Dx treatments (figs. 6.2b; 6.3a)

When PTH treatment was delayed, the number of colonies was considerably decreased in both the 10nM and 100nM cultures (fig. 6.2c; 6.3b).
In the PTH+Dx cultures a similar significant decrease was seen in the 10nM and 100nM Dx treatments (figs. 6.2d; 6.3b)

6.3.4: Mean Colony Area.

Treatment with PTH had no significant effect on mean colony area under any condition (fig. 6.4a,b).

6.3.5: Total Colony Area.

Using a single representative plate from each treatment group, where PTH was applied continuously, the total colony area did not differ greatly between the control and the treatment groups (fig. 6.5a). In cultures where PTH was combined with Dx, a considerable decrease in total area was seen in the 10nM Dx cultures, $\approx$ x 0.5 compared to control. A slight decrease was seen in the 100nM Dx cultures (fig. 6.5a).

Irrespective of the presence of absence of Dx, when PTH treatment was delayed, the total colony areas were considerably reduced in a dose dependent manner, compared to the control (fig. 6.5b).

6.3.6: AP+ Colonies.

Continuous treatment with PTH at both 10nM and 100nM caused a significant increase ($p < 0.05$) in the number of colonies expressing AP compared to the control (figs. 6.6a; 6.7a). PTH further supplemented with Dx significantly increased ($p < 0.05$) the number of colonies expressing AP in the 100nM Dx treatment (figs. 6.6b; 6.7a).

Delayed treatment with PTH at the 100nM level caused a significant reduction ($p < 0.05$) in the number of AP+ colonies (figs. 6.6c; 6.7b).
In Dx supplemented cultures, treatment with PTH at both 10nM and 100nM caused significant decreases ($p < 0.05$) in the number of AP+ colonies (figs. 6.6d; 6.7b).

Comparing early and late treatments revealed that the number of AP+ colonies was lower in all the late PTH treatments, and significantly so in the 100nM, 10nM Dx and 100nM Dx cultures ($p < 0.05$) (figs. 6.6c,d; 6.7a,b).

6.3.7: AP+ Colony Mean Area.

Treatment with PTH±Dx, irrespective of conditions, had no significant effect on the mean areas of AP+ colonies (figs. 6.8a,b).

6.3.8: AP+ Colony Total Area.

Cultures treated continuously with PTH had greater colony total areas than the control (fig. 6.9a). In Dx supplemented cultures, the 10nM treatments had a reduction in

colony area while the 100nM treatments had a considerable increase in area (fig. 6.9a)

Irrespective of the presence of absence of Dx, when PTH treatment was delayed, the total colony areas were considerably reduced in a dose dependent manner, compared to the control (figs. 6.9b).

6.3.9: Percentage of Colonies Expressing AP.

Continuous or delayed treatment with PTH±Dx had no effect on the percentage of colonies expressing AP (fig. 6.10a,b). In the continuously treated, the number of AP+ colonies correlated with the total number of colonies, adj. $r^2 = 0.61$, ($p < 0.05$) In the delayed treated, the number of AP+ colonies correlated with the total number of colonies, adj. $r^2 = 0.70$, ($p < 0.05$)

Comparison between the continuous and the late treatments, revealed no significant differences in the percentage of AP+ colonies (figs. 6.10a,b).

6.3.10: Flow Cytometry.

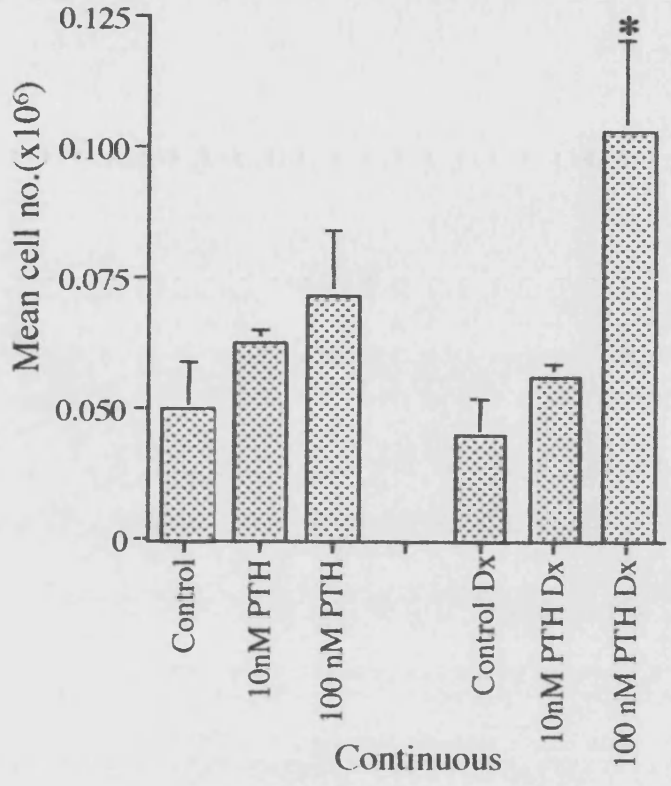
In cultures continuously treated with 10nM PTH, the sub-population distributions were markedly different from the control (fig. 6.11c,d; simplified in fig. 6.15a). The percentage of cells in 10nM treatment was either ≅ double or ≅ half of the comparable control sub-population. Control:Treatment; STRO-1-/AP-, 24:51; STRO-1+/AP-, 45:18; STRO-1+:AP+, 21:11; STRO-1-/AP+,10:20. In the 100nM cultures the differences from the control were not as marked as that seen in the 10nM treatments (figs. 6.11b,d; 6.15a); STRO-1-/AP-, 29; STRO-1+/AP-, 31; STRO-1+:AP+, 24; STRO-1-/AP+, 16. Compared to the controls, the PTH treated cultures had a higher percentage of cells in the STRO-1-/AP+ and STRO-1-/AP-populations.

In the continuous PTH Dx treated cultures, the sub-population distributions in the PTH treated cultures were markedly shifted towards the osteogenic phenotype (fig. 6.12c,d; 6.15b). Compared to the controls, the PTH Dx treated cultures had a higher percentage of cells in the STRO-1-/AP+ and a lower percentage in the STRO-1-/AP-populations.

Where PTH treatment was delayed, the overall sub-population distributions were notably different from the control (fig. 6.13b-d; 6.16a). In both the 10nM and 100nM cultures, the distribution of cells was skewed towards the less differentiated phenotypes. In the STRO-1-/AP+ population, the percentage of cells in the PTH groups was considerably lower than the control.

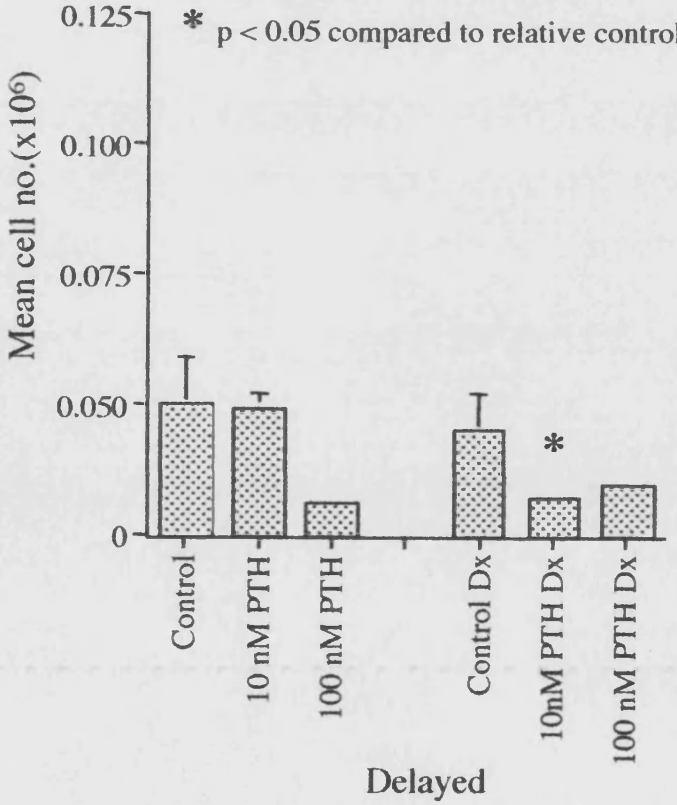
In these cultures, when PTH was supplemented with Dx, a population pattern similar to that seen in the -Dx cultures was observed (fig. 6.14b-d; 6.16b). The Dx supplemented system differed mainly in the proportion of cells present in the 100nM STRO-1-/AP- and STRO-1+/AP- populations.

Figure 6.1: Effect of PTH±Dx Treatment on Cell Number.
(a) Continuous and (b) Delayed.



(a)

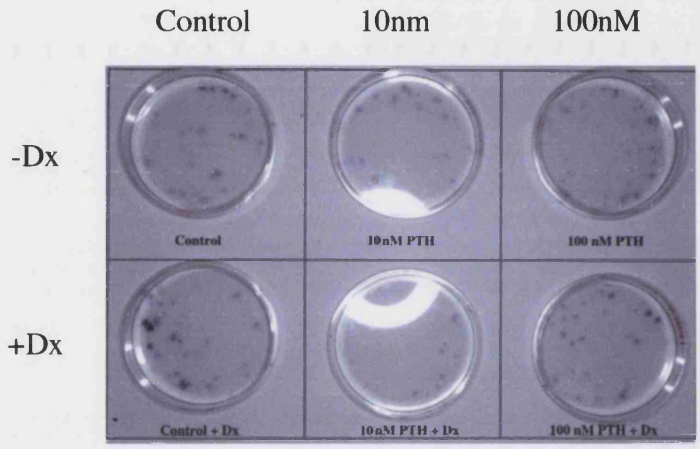
Error bars represent S.E. of mean; n = 4;
* $p < 0.05$ compared to relative control



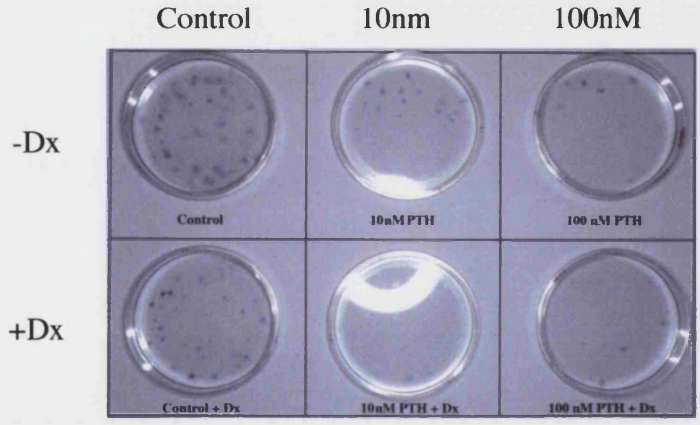
(b)

Figure 6.2: Photographs showing colony coverage at 21 days
in response to continuous treatment with (a) PTH; (b) PTH Dx.
and delayed treatment with (c) PTH; (d) PTH Dx.

Figure 6.2: Effect of PTH±Dx Treatment on Colony Number, (a,b) Continuous and (c,d) Delayed.

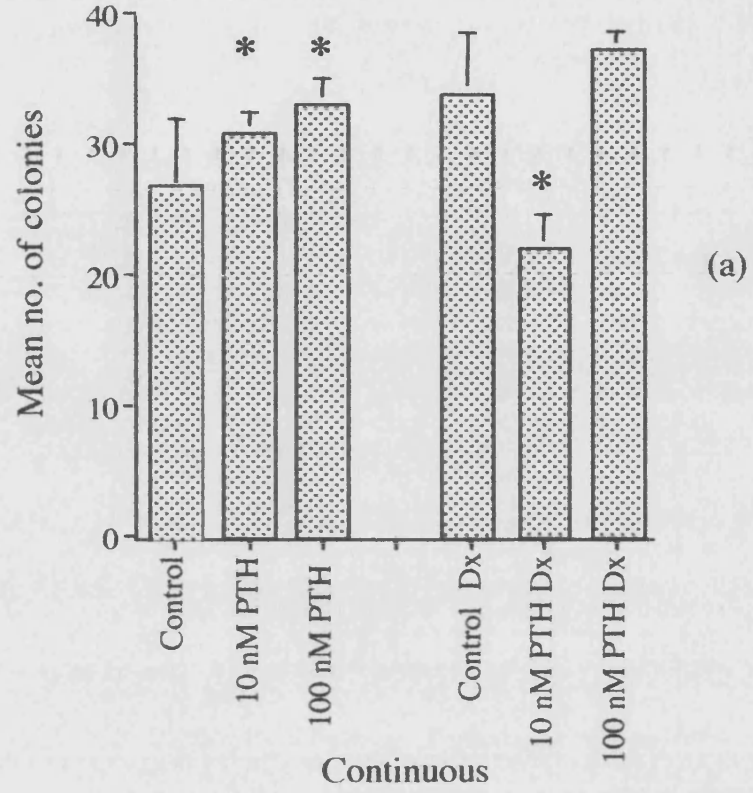


Continuous

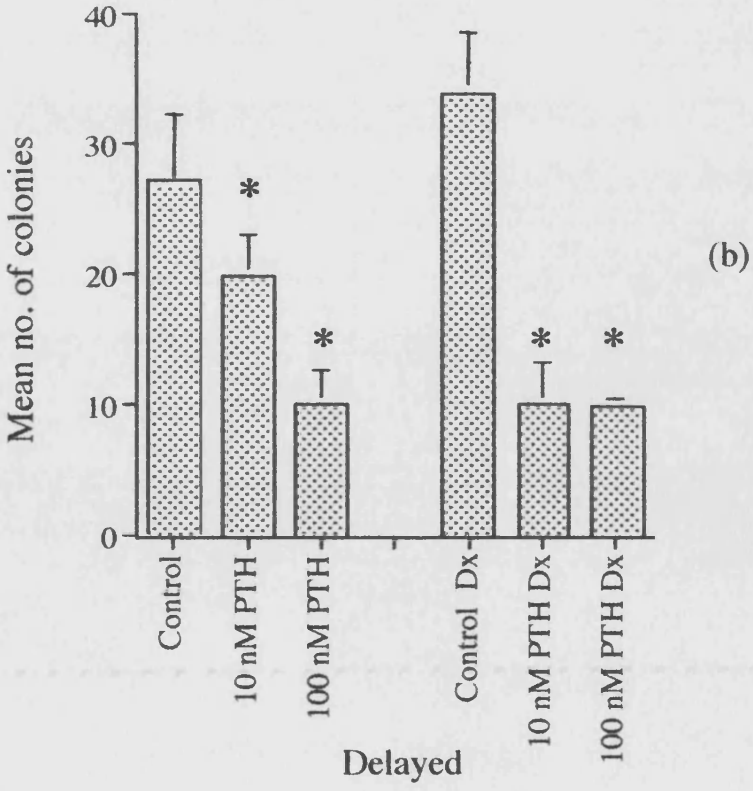


Delayed

Figure 6.3: Effect of PTH±Dx Treatment on Colony Number, (a) Continuous and (b) Delayed.

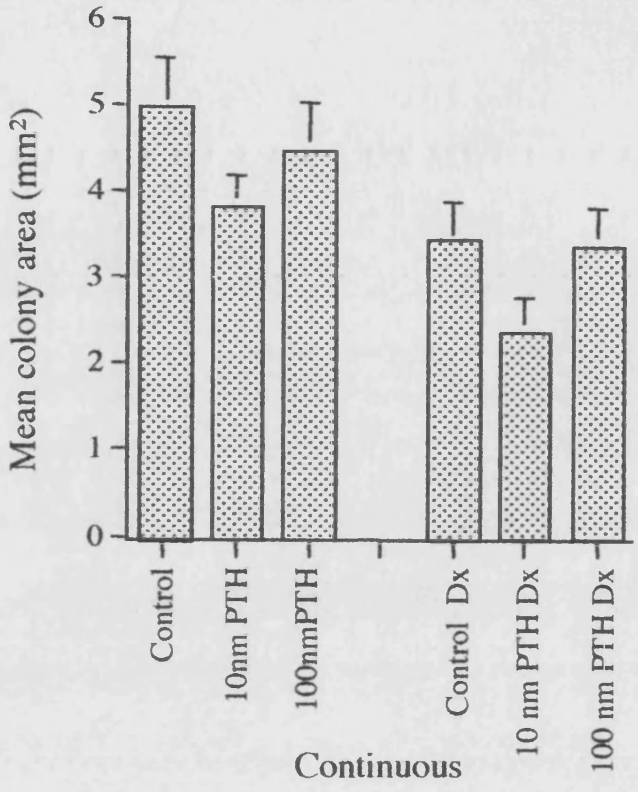


(a)

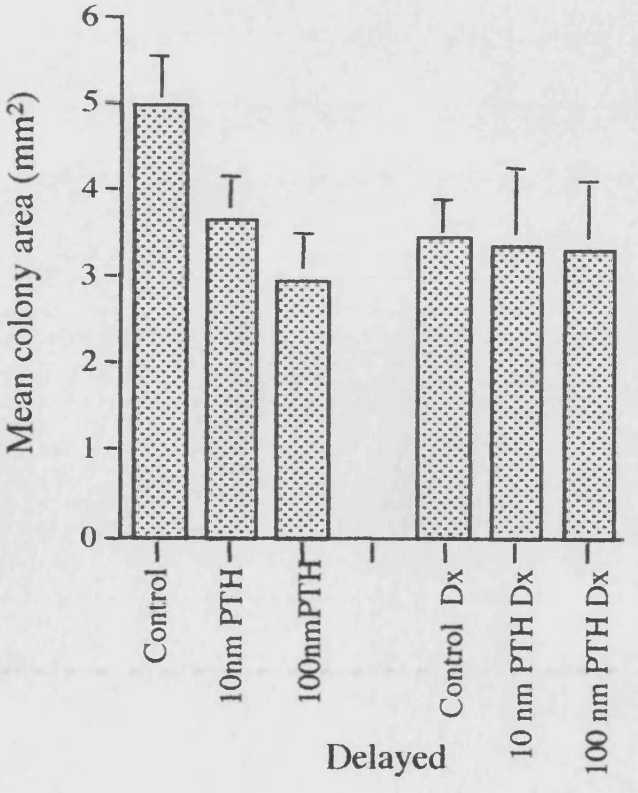


(b)

Figure 6.4: Effect of PTH Treatment on Mean Colony Area, (a) Continuous and (b) Delayed.

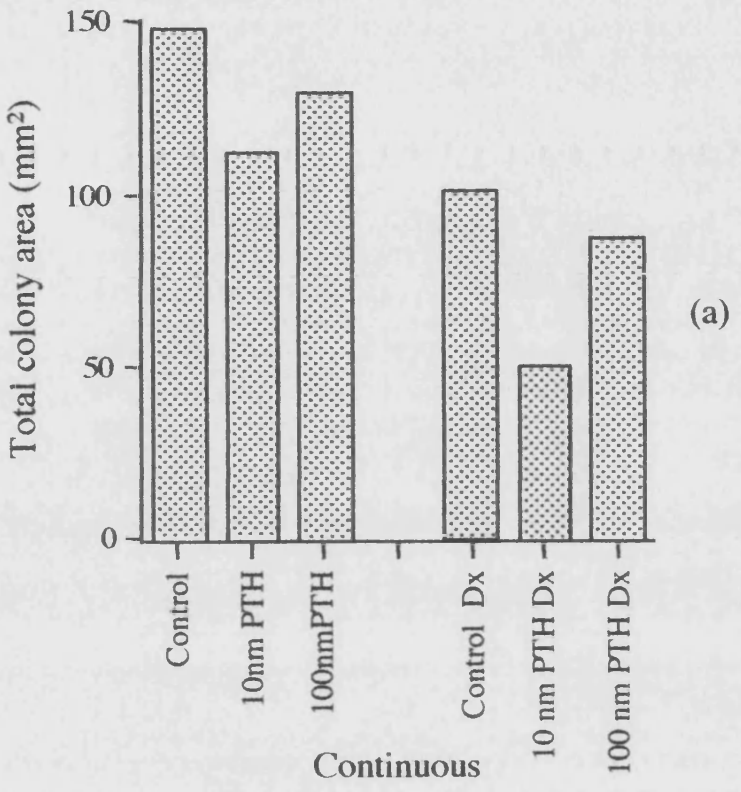


(a)

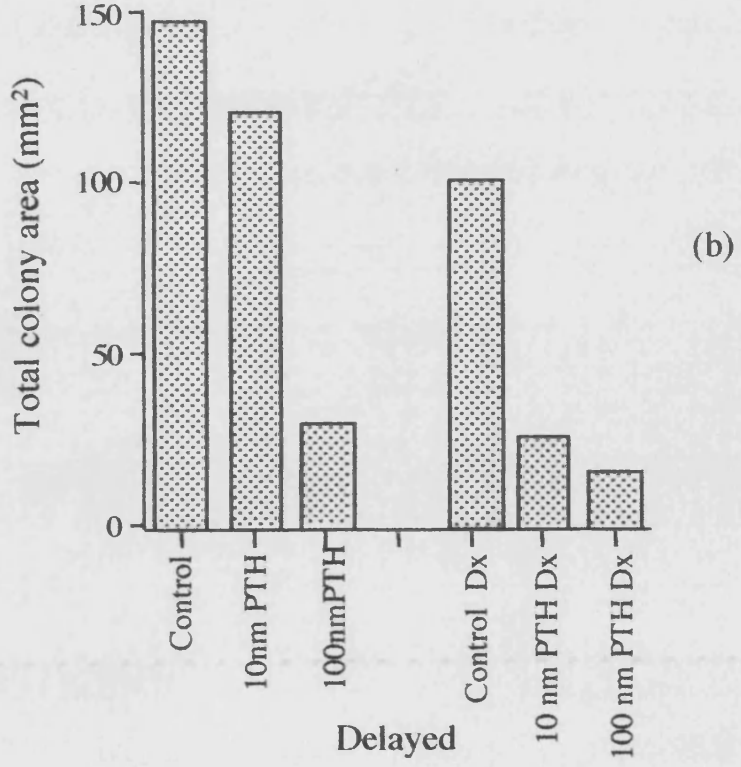


(b)

Figure 6.5: Effect of PTH Treatment on Total Colony Area, (a) Continuous and (b) Delayed.



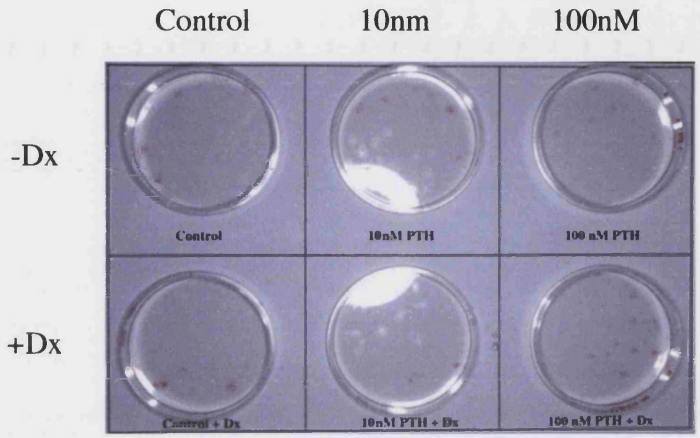
(a)



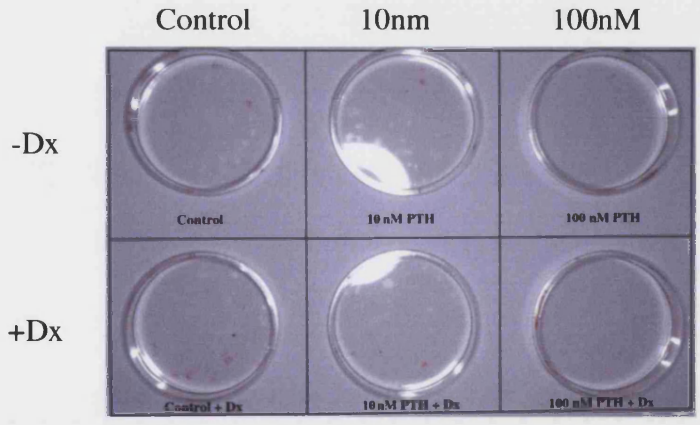
(b)

Figure 6.6: Photographs showing AP+ colony coverage at 21 days in response to continuous treatment with (a) PTH; (b) PTH Dx. and delayed treatment with (c) PTH; (d) PTH Dx.

Figure 6.6: Effect of PTH±Dx Treatment on AP+ Colony Number, (a,b) Continuous and (c,d) Delayed.

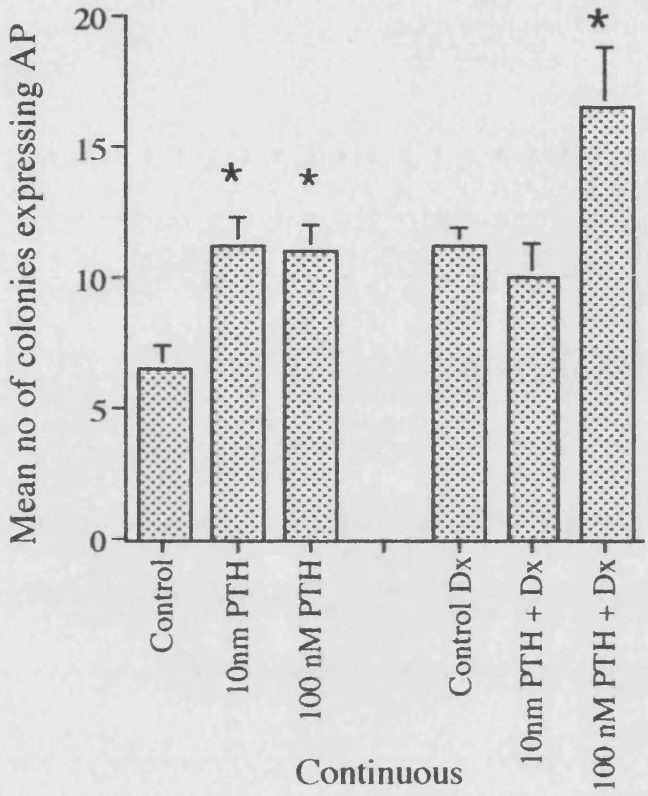


Continuous

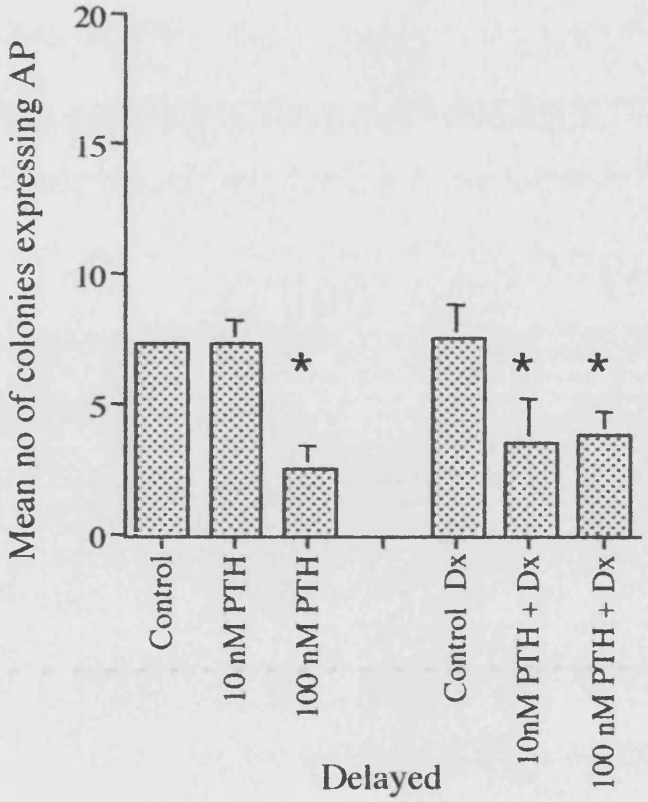


Delayed

Figure 6.7: Effect of PTH±Dx Treatment on AP+ Colony Number, (a) Continuous and (b) Delayed.



(a)



(b)

Figure 6.8: Effect of PTH±Dx Treatment on AP+ Mean Colony Area, (a) Continuous and (b) Delayed.

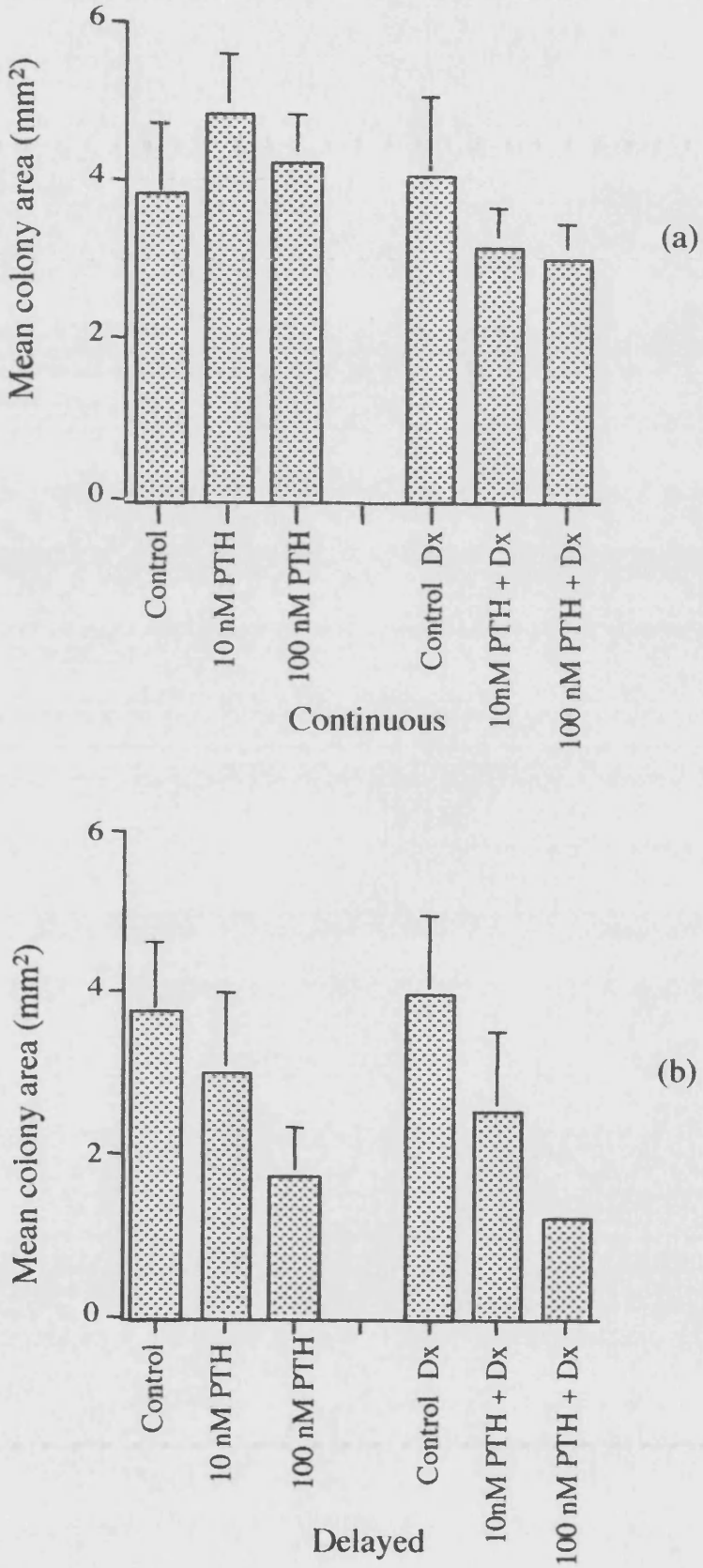
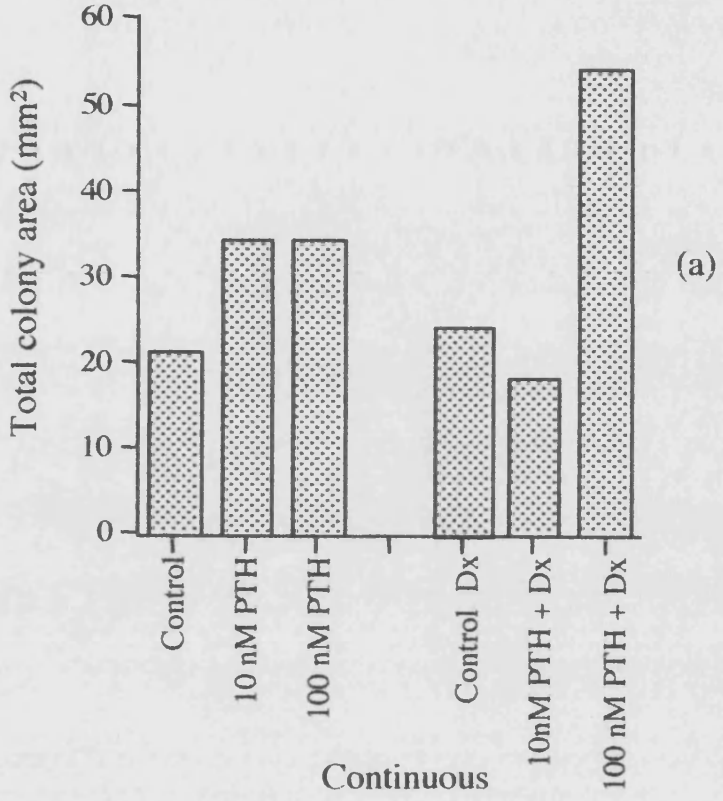


Figure 6.9: Effect of PTH±Dx Treatment on AP+ Total Colony Area, (a) Continuous and (b) Delayed.



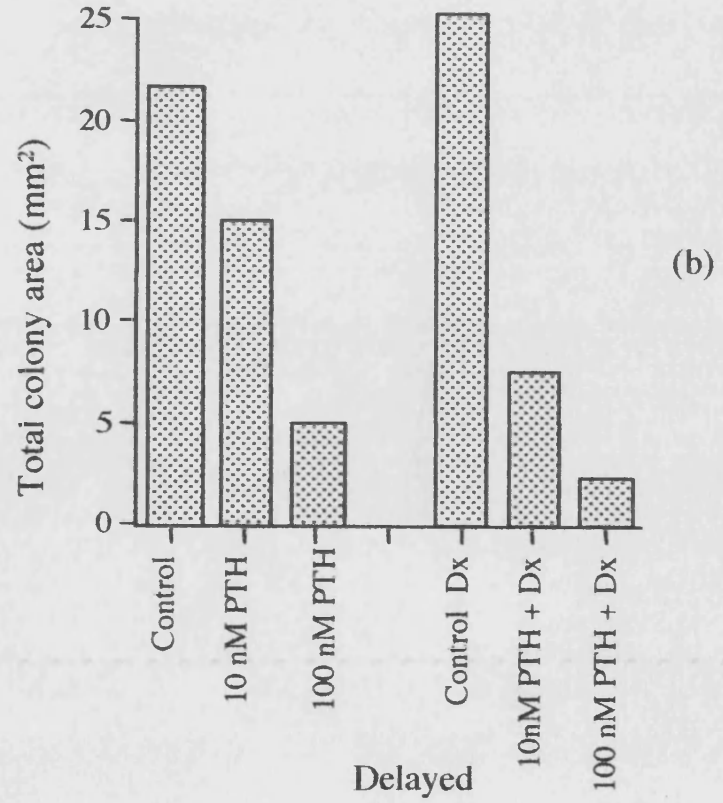
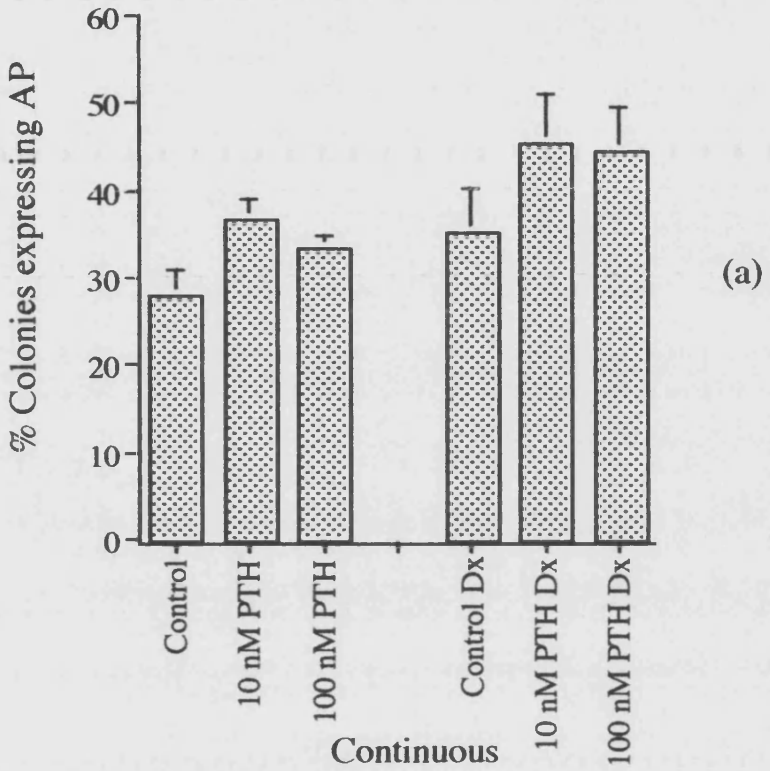


Figure 6.10: Effect of PTH Treatment on Percentage of Colonies Expressing AP, (a) Continuous and (b) Delayed .



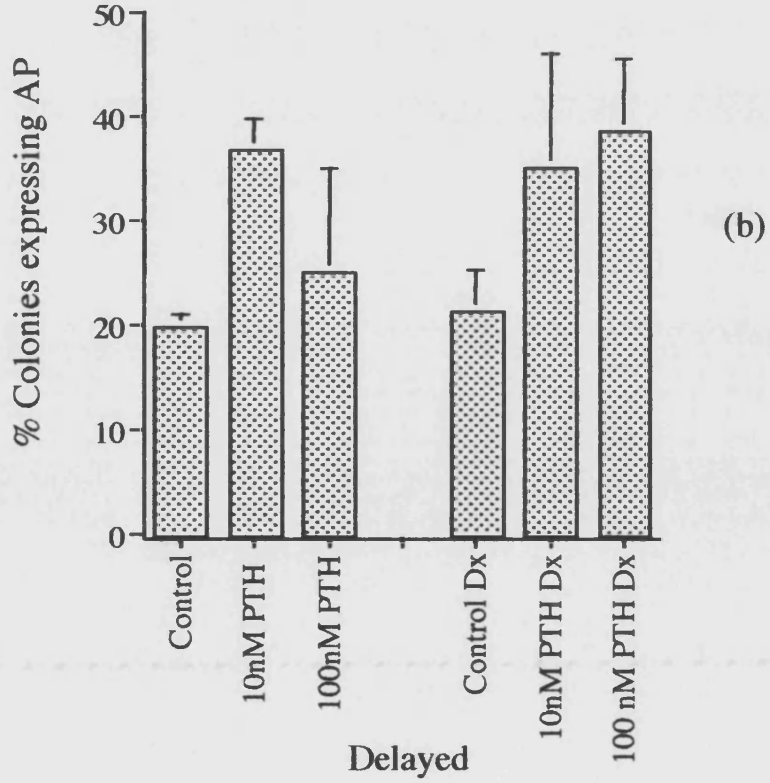
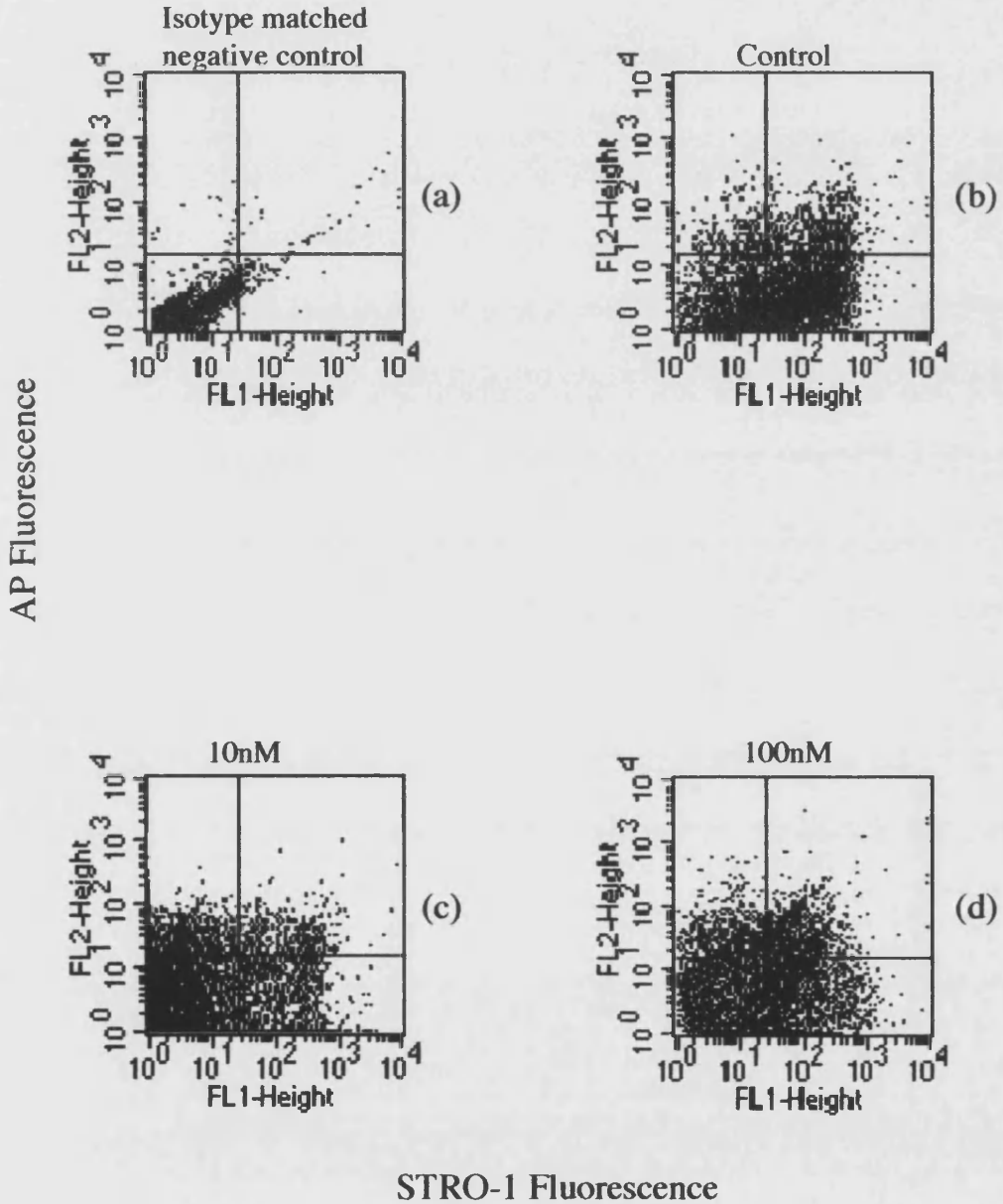
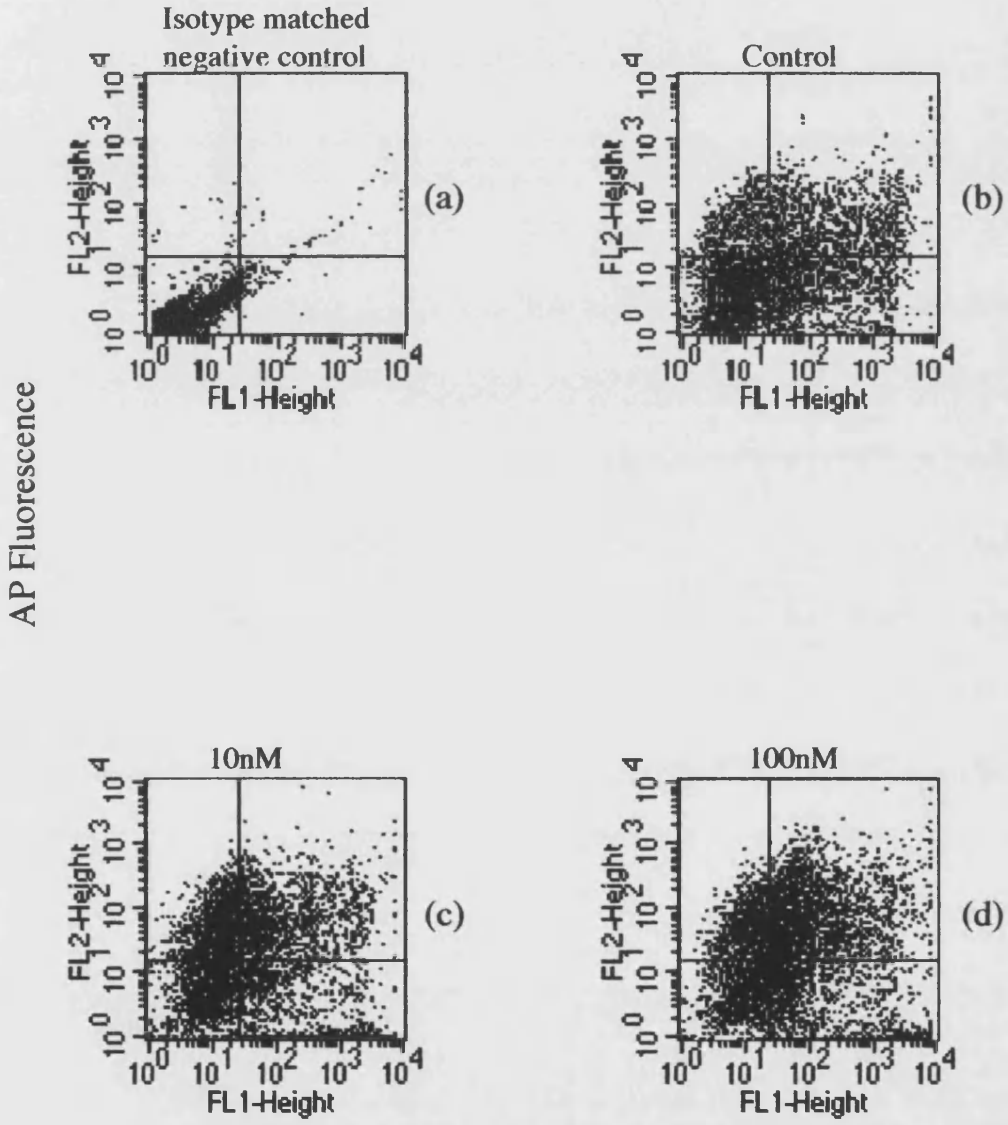


Figure 6.11 (a-d): Effect of Continuous PTH Treatment on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.



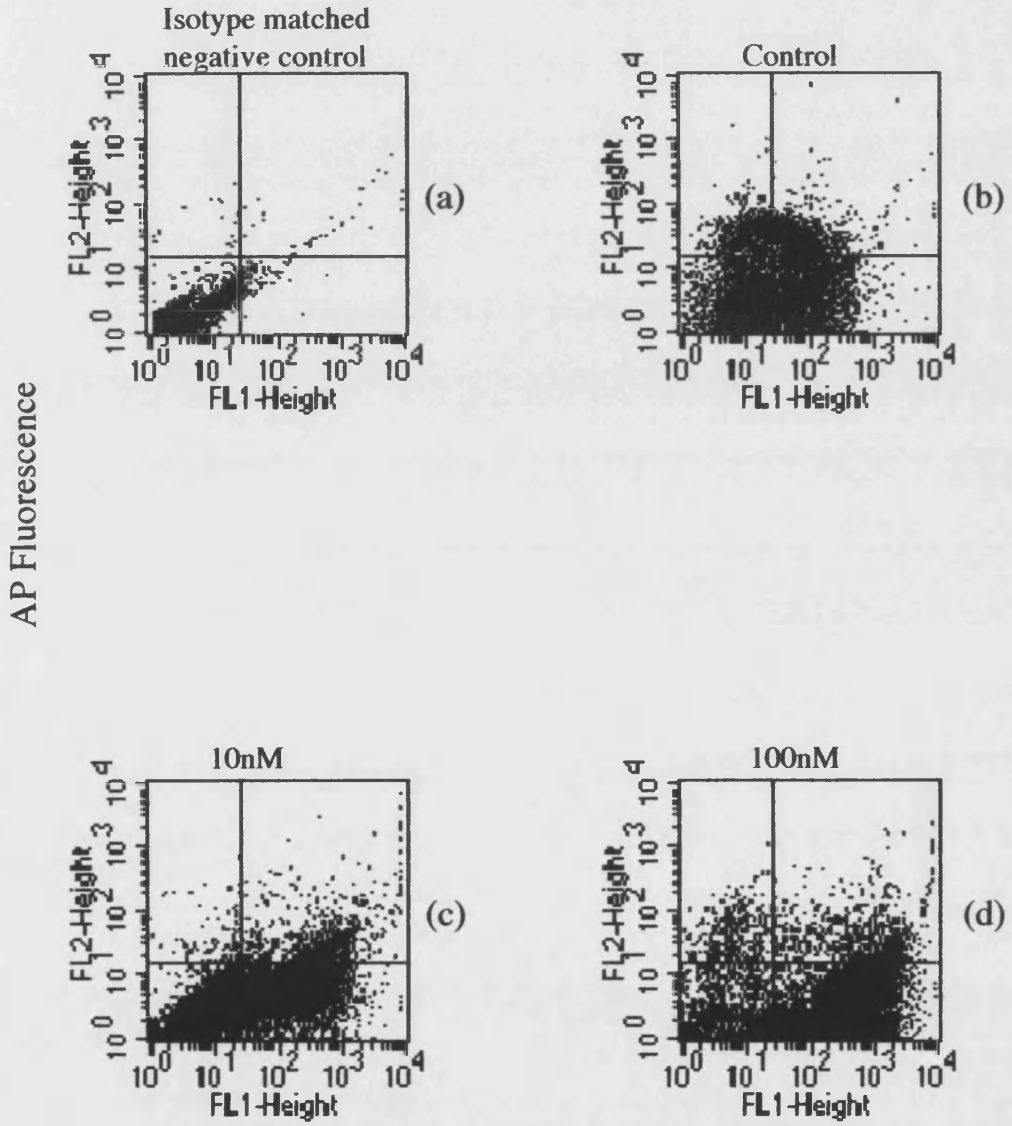
Cells that had been dual-labelled for expression of STRO-1 and AP antigens were visualised as dot plots with green fluorescence (FITC) on the *x* -axis and red fluorescence(R-PE) on the *y*--axis. Quadrants were set using the negative control sample such that >95% of cells appeared within the lower left quadrant (a).

Figure 6.12 (a-d): Effect of Continuous PTH Dx Treatment on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.



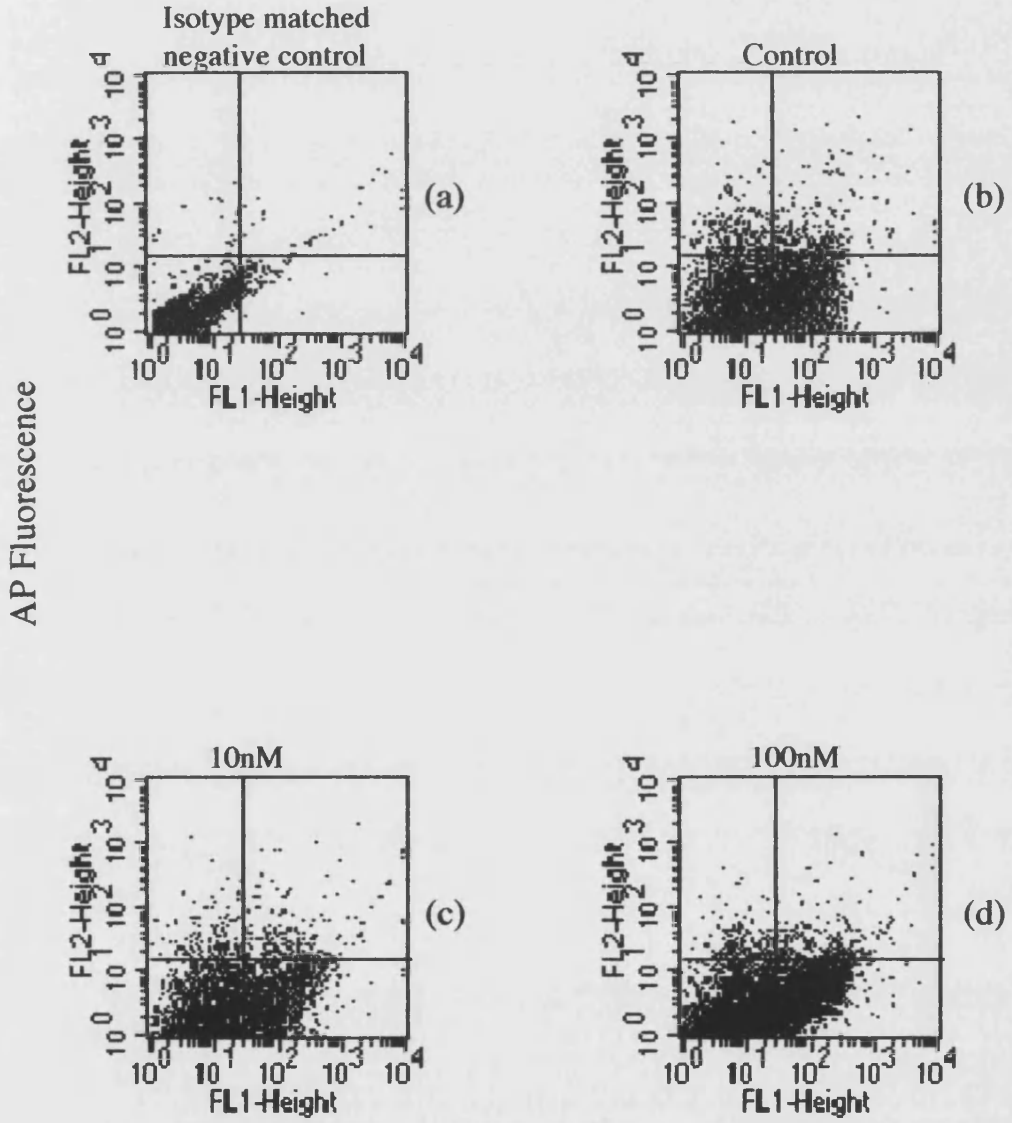
Cells that had been dual-labelled for expression of STRO-1 and AP antigens were visualised as dot plots with green fluorescence (FITC) on the *x* -axis and red fluorescence(R-PE) on the *y*--axis. Quadrants were set using the negative control sample such that >95% of cells appeared within the lower left quadrant (a).

Figure 6.13 (a-d): Effect of Delayed PTH Treatment on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.



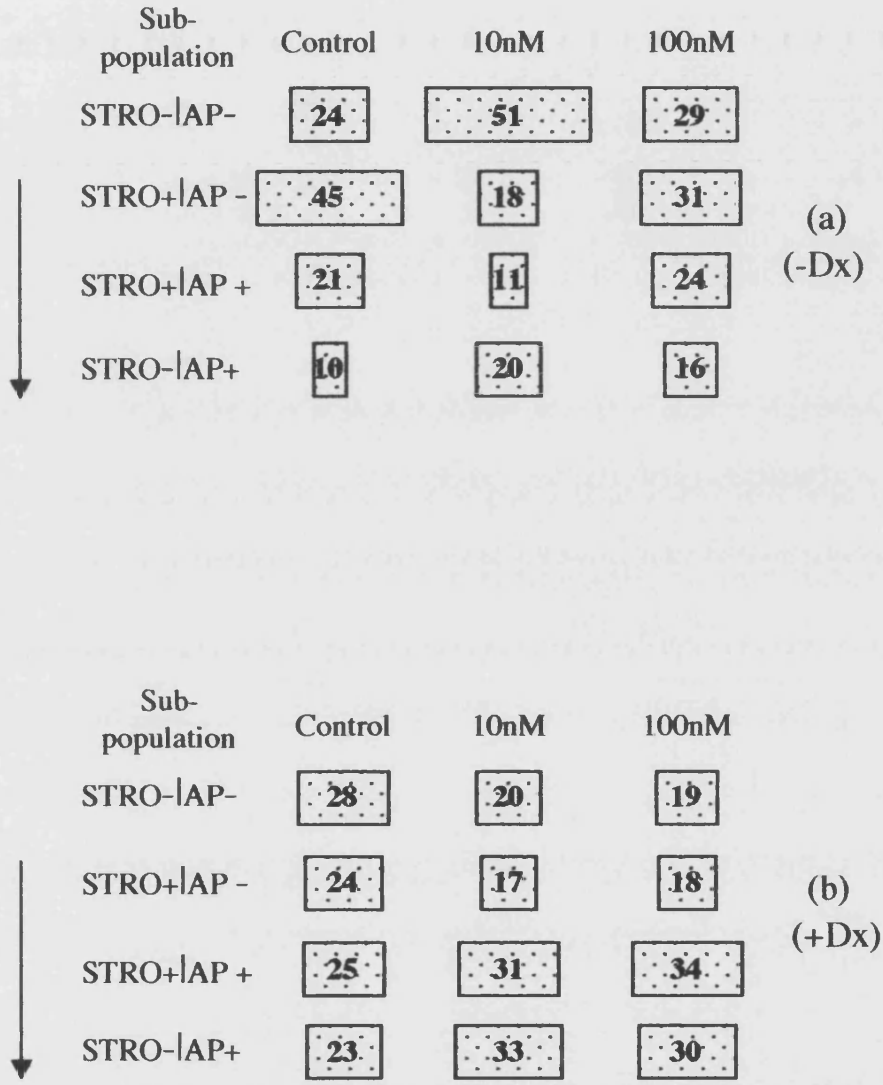
Cells that had been dual-labelled for expression of STRO-1 and AP antigens were visualised as dot plots with green fluorescence (FITC) on the *x* -axis and red fluorescence(R-PE) on the *y*--axis. Quadrants were set using the negative control sample such that >95% of cells appeared within the lower left quadrant (a).

Figure 6.14 (a-d): Effect of Delayed PTH Dx Treatment on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.



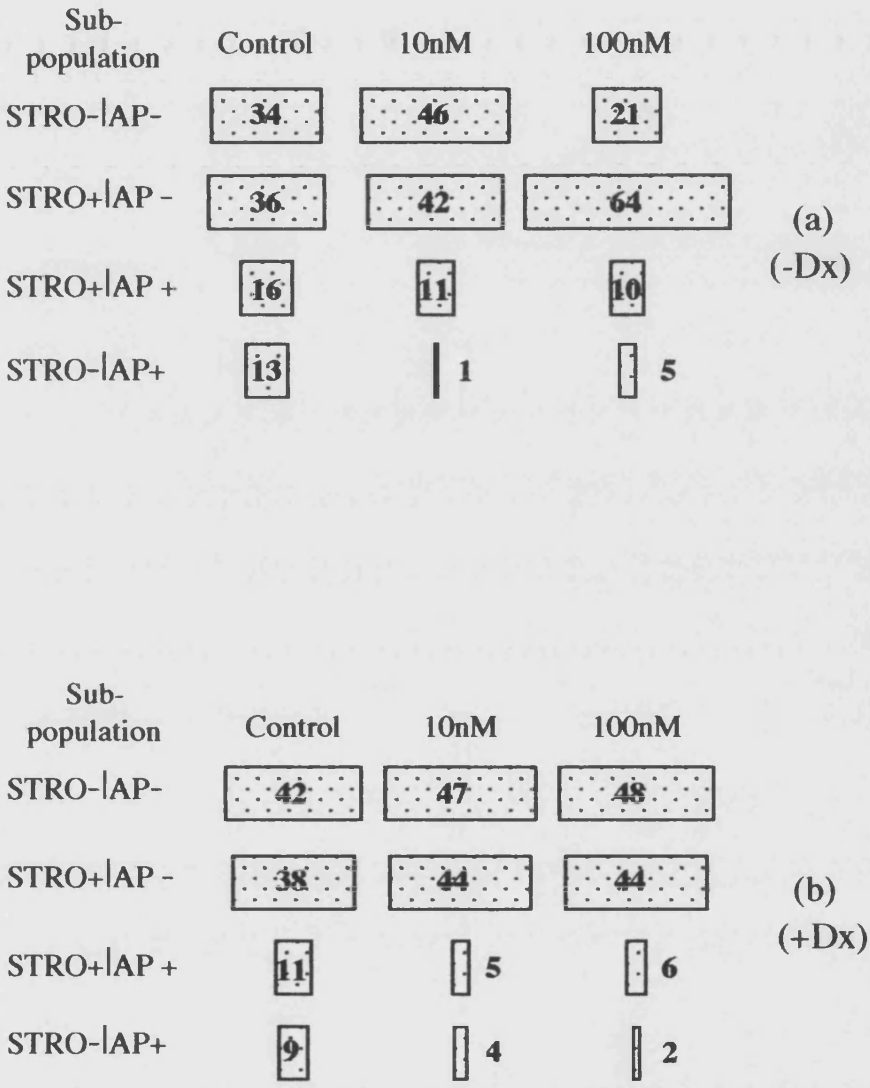
Cells that had been dual-labelled for expression of STRO-1 and AP antigens were visualised as dot plots with green fluorescence (FITC) on the *x* -axis and red fluorescence(R-PE) on the *y*--axis. Quadrants were set using the negative control sample such that >95% of cells appeared within the lower left quadrant (a).

Figure 6. 15: Effect of Continuous (a) PTH (b) PTH Dx Treatment on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.



Relative proportions of sub-populations of cells labelled with the STRO-1 and AP antibodies. Block areas represent relative sub-population sizes, numbers associated with blocks are percentage of cells (to nearest percent). Arrows represent the direction of increasing osteogenic differentiation.

Figure 6.16: Effect of the Delayed Treatment of (a) PTH (b) PTH Dx on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.



Relative proportions of sub-populations of cells labelled with the STRO-1 and AP antibodies. Block areas represent relative sub-population sizes, numbers associated with blocks are percentage of cells (to nearest percent). Arrows represent the direction of increasing osteogenic differentiation.

6.4: Discussion.

Continuous treatment with PTH at 10nM and 100nM concentrations caused early changes in bone marrow stromal cell morphology similar to that seen in the initial PTH experiment. However when PTH was applied late, no meaningful difference in cell morphology was noted between the late treatments and the control. This suggests that PTH must be in the culture system early in order to affect cell morphology. Late PTH treatments were applied after the non-adherent cell population had been removed and the finding of this experiment lends support to the notion that the primary target cell of PTH is the uncommitted/non-adherent cell[289].

The results of this experiment also support the suggestion that the target cell may be the immature osteoblast[294] or pre-osteoblast[296]. In the present system, the late application of PTH allows such cells to progress to a more advanced stage of differentiation before being challenged by PTH. This therefore reduces the number of potential targets for PTH in the culture and consequently reduces the effect of the treatment.

Treatment with PTH at 10nM and 100nM concentrations caused no significant change in cell numbers. This finding is at odds with that of the initial experiment which found that the 100nM treatment was associated with a significant increase in cell numbers. This discrepancy may be due to an inter-donor variation caused by differences in age (69:31) or sex (F:?).although this concept is not supported by recent findings suggesting that the proliferative potential of BMSC declines with age and that gender has no effect[297].

Examination of the number of cells in the control cultures of both experiments reveals no significant difference[1] suggesting donor age is not a major influence on cell proliferation in this case. It is possible that an age related response to PTH could be responsible for the difference. However, this is not supported by the findings of the work of Dr Sue Walsh[1] who found no age-related correlation with cAMP response. Evidence from a rat marrow[298] and human trabecular explant model[299] suggesting that PTH induced cAMP responses are *reduced* with age, only serves to confound the issue.

Where PTH was supplemented with Dx, a dose dependent increase in cell numbers was seen and this increase was significant in the 100nM Dx treatments. This is

[1] Unpublished results from this lab.

similar to the effect seen in the comparable treatment groups in the initial PTH dose response experiment. The Control Dx and 10nM Dx treatments had fewer cells than the comparable -Dx cultures which is in accordance with the proliferative inhibition associated with Dx treatments[180]. The 100nM Dx treatment had more cells than the comparable -Dx and this may be due to synergy associated with the level of PTH applied.

When PTH was applied late, the number of cells in each of the treatment groups was lower than that of the comparable continuously treated and significantly so in the 100nM groups.

This experiment does not provide evidence to support the concept that PTH, when present from the start of culture, increases the number of cells. However it does suggest that its late application has an inhibitory effect on cell numbers. This is supported by evidence that PTH exerts an inhibitory effect on osteogenic cells that is positively related to the differentiation stage[294]. PTH stimulation of proliferation in cultured mouse calvaria reverts to inhibition when the cells reach an osteoblastic phenotype[144]. PTH has also been shown to increase proliferation in cells associated with trabecular bone, and when these cells were isolated and grown *in vitro*, after 12 hours the effect of PTH had become inhibitory[300].

The late application of PTH Dx caused a significant reduction in the number of cells in both the 10nM Dx and 100nM Dx cultures. This supports the idea that the increases in cell numbers which are associated with the continuous application of PTH Dx are due to an influence on early events. Furthermore this finding provides further evidence that PTH inhibits cell proliferation in later stages of culture.

The effects of PTH on bone cell numbers can be summarised in the form of a simple model. PTH recruits cells from the non-adherent/uncommitted population and promotes them to adherent, committed cell types. This initially increases the number of cells in the system. These cells proliferate and proceed along the differentiation pathway and, with increasing differentiation the inhibitory influence of PTH is also increased. When PTH is applied early, the recruitment of non-adherent/uncommitted cells goes some way to compensate for the inhibitory effects of PTH and, dependent on how long the cells spend in culture, can result in an increase in numbers. The late application of PTH does not provide the opportunity to recruit additional cells so the main effect is inhibitory and results in a reduction in cell numbers.

Treatment with hPTH at 10nM and 100nM significantly increased the numbers of colonies compared to the control. This is similar to the findings of the PTH dose response experiment, where the 10nM treatment was also significantly higher than the control while the 100nM although higher, was not significantly so. The present experiment also yielded more colonies for a given treatment group than the initial dose response experiment, this may be a result of inter-donor sex/age differences although the findings of recent research does not support this concept[298].

Treatment with PTH Dx was associated with a significant reduction in the number of colonies in the 10nM Dx cultures and a small insignificant rise in the 100nM Dx group. This compares to the previous experiment where a small increase was seen in the 10nM Dx cultures and a larger significant rise in the 100nM Dx.

Late treatments with PTH caused a significant reduction in the number of colonies compared to the control. This was also true in PTH Dx groups. Comparing the late treatments with the continuous reveals a reduction in the number of colonies in all the late treated groups. This deficit was significant in the 100nM, 10nM Dx and the 100nM Dx treatments.

These findings are in accordance with the idea that treatment with PTH enhances the recruitment of a non-adherent/uncommitted population and that PTH must be present early to achieve this. These results also suggest that the presence of PTH in later stages of culture have an inhibitory effect on the development of colonies. This effect is unlikely to be due to an inhibition of initial adherence/recruitment of cells as PTH is not present at that stage. The influence could be mediated via an inhibition of proliferation i.e. when a proportion of adherent cells with the potential to develop into colonies are inhibited or arrested in growth by the presence of PTH.

An indication as to the proliferative potential of a colony is provided by the colony area[180] and density. In the late treated PTH cultures the dose dependent reduction in mean colony area, although statistically insignificant, support the hypothesis that the late application of PTH inhibits colony proliferation. The same effect is not seen when Dx is added to the system, this could be because the inhibitory effect Dx has on colony proliferation[180] masks the effects of the PTH.

As the total colony area of a culture is a function of mean colony size and number, the total colony areas in the cultures where PTH±Dx is added late are markedly reduced in a dose dependant manner.

Treatment with PTH±Dx caused a significant increase in the number of AP+ colonies in a manner similar to that seen in the initial PTH dose response experiment. As in the previous experiment this increase is mainly, but not entirely, due to the presence of a greater number of colonies in these groups. The level of correlation between these parameters suggests that, to a lesser extent, other influences are involved in this increase. It is therefore possible that PTH treatment also enhances osteogenic differentiation.

In cultures where PTH±Dx was applied late, the number of AP+ colonies were equal to or significantly less than the controls. This decrease in AP+ colonies is primarily related to the number of colonies in cultures although to a lesser extent, other influences such as inhibition of osteogenic differentiation might be involved. The proliferative potential of AP+ colonies is inhibited by the late application of PTH as evidenced by the reduction in mean colony area. This deficit acts in concert with the reduced number of AP+ colonies in these cultures to bring about the marked dose dependant reduction in total AP+ colony area.

Although the correlation between the total number of colonies and the number of AP+ colonies suggests that the latter is primarily a function of the former, the coefficient value suggested that other lesser influences were also involved. Analysis of the cell surface expression of the STRO-1 and AP antigens reveals that treatment with PTH is associated with a small increase in the percentage of STRO-1-/AP+ cells. When Dx is present in this system the proportion of these differentiated cells is increased. Late treatment with PTH reverses these effects. This suggests that early PTH treatment is enhancing osteogenic differentiation as evidenced by increased proportions of STRO-1-/AP+ cells and it is likely that the increase in AP+ colonies is partly due to this effect. It also provides evidence that the late administration of PTH inhibits osteogenic differentiation and contributes to the reduction of AP+ colonies seen in these groups.

Previously discussed work[294,296] argues against PTH having a direct osteogenic influence on cells in the later stages of the osteoblast lineage. It may be that PTH recruitment of non-adherent/committed cells preferentially favours cells with greater osteogenic potential, thereby increasing their proportions in culture. Either way, the evidence provided by this experiment, and that of work already discussed, is not sufficient to define a mechanism whereby cultures exposed early to PTH have increased numbers of AP+ colonies and STRO-1-/AP+ cells.

6.5: Summary.

Comparison of the results from controls, continuous and late treatments give support to the notion that PTH is required early in culture to bring about positive osteogenic changes. This is evidenced by increased numbers of AP+ colonies and proportions of STRO-1-/AP+ cells in the continuous treatments. The reduction in these parameters and in virtually all those measured in the late treated groups provides additional evidence suggesting that late treatment with PTH has an inhibitory effect on osteogenic differentiation in this system. This finding encourages an investigation into the effects of early PTH treatments on BMSC.

Chapter 7: The *In Vitro* Effects Of Early Parathyroid Hormone Treatments On Bone Marrow Stromal Cells.

7.1: Introduction.

The initial PTH dose response experiment demonstrated that continuous treatment with PTH at concentrations of 10nM and 100nM is capable of increasing the number of colonies, cells and when applied with Dx, AP+ colonies in cultures of human BMSC. This suggests that PTH targets early (uncommitted) cells and affects their adhesion and/or the recruitment to their osteogenic lineage. The subsequent experiment in which PTH treatments were applied late reaffirmed this possibility and provided additional information that suggested late treatment with PTH is associated with an inhibition of osteogenic differentiation.

On the basis of these findings it was postulated that the positive effects of PTH on osteogenic differentiation depend on the hormone being present at the earliest stages of culture and that its presence at later stages is associated with an inhibition of this process. To test this hypothesis, experiments were performed in which PTH was applied from the start of the culture period (continuous) and also applied to the culture for the initial 48 hours only (early). The rationale for this regime is, in the early system, the suggested positive effects of PTH treatment on the recruitment of non-adherent/uncommitted cells will not be attenuated by the negative effects associated with its presence late in culture.

7.2: Methods.

Bone marrow cells were obtained as described previously, from segments of rib removed from a male patient aged 66, during routine thoracic surgery at Frenchay Hospital, Bristol. Cells were plated at a density of 2 x $10^5/cm^2$ and cultured for 21 days in standard media formulation supplemented with 100μM ascorbate-2-phosphate with or without hPTH(1-34) at 10nM and 100nM and in the presence or absence of Dx.

The following parameters were assessed using cells seeded into T75 flasks (X4/treatment), cell numbers, surface expression of STRO-1 and AP and the expression of mRNA transcripts for OC, BSP CBFA1 and GAPDH. To estimate the effects on colony forming efficiency (CFE), and colony area, cells were seeded in petri dishes (4 petris/treatment).

To investigate the interaction between PTH and Dx, bone marrow cells were obtained as described previously, from segments of rib removed from 3 patients (2 male, 1 female) age range 52-73 (mean 65 during routine thoracic surgery at Frenchay

Hospital, Bristol. Cells were plated at a density of 2 x $10^5/cm^2$ and cultured for 21 days in standard media formulation supplemented with 100μM ascorbate-2-phosphate with or without hPTH(1-34) at 10nM and in the presence and absence of Dx.

To assess the effects of Dx on the expression of the PTH receptor (PTHr1) by flow cytometry, cells were seeded (2 x $10^5/cm^2$) in T75 culture flasks (x4/treatment/analysis) and cultured for 21 days. For the measurement of intracellular cAMP levels, cells were plated in 6 well plates overnight (x1 plate/treatment).

For both experiments, cells were cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2. After 48 hours, the non-adherent cells were removed, the cultures were replenished with appropriate medium and fed twice weekly thereafter. At the appropriate time point, the cells were harvested and parameters assessed according to methods described in Chapter 2.

All experiments were repeated at least once and data from a single representative experiment is shown. The distribution of the data was confirmed as normal using the Shapiro Wilk W test, where data was not normally distributed, a log transformation was used to normalise the distribution prior to test. Differences in the data were assessed using the one way Analysis of Variance followed by the Tukey-Kramer test of Honest Significant Difference ($p < 0.05$).

7.3: Results.

7.3.1: Cell Morphology.

The continuous presence of PTH affected the morphology of the cells in a similar manner to that seen in the previous experiments. These changes were also apparent in cultures where PTH was applied early. Dx produced changes which were similar to those seen in the previous experiments.

7.3.2: Cell Numbers.

In the absence of Dx, continuous treatment with PTH did not effect cell number (fig. 7.1a). In the presence of Dx, treatment with PTH was associated with a dose dependent increase in cell number, $p < 0.05$ at 100nM. When PTH was applied early, a small insignificant dose dependent increase in numbers was seen (fig. 7.1b). In the PTH+Dx groups a similar increase was noted and this was significant in the 100nM Dx cultures. Comparing the continuous and early treatments revealed no significant differences in cell numbers

7.3.3: Total Number of Colonies.

Continuous treatment with PTH increased the number of colonies in a dose dependent manner although this increase was not significant (figs. 7.2a; 7.3a). In cultures treated with PTH+Dx a significant increase ($p < 0.05$) in the number of colonies was seen in the 100nM Dx group (figs. 7.2b; 7.3a). When PTH was applied early, the number colonies was significantly increased ($p < 0.05$) in a dose dependent manner (figs. 7.2c; 7.3b). This was also true of cultures treated with PTH+Dx (figs. 7.2d; 7.3b).

7.3.4: Mean Colony Area.

Continuous treatment with PTH±Dx did not significantly effect on the mean area of the colonies (fig. 7.4a). In the early treatments, where Dx was added, a dose dependent increase in mean colony area was significant in the 100nM Dx cultures (fig. 7.4b).

7.3.5: Total Colony Area.

Continuous PTH treatment was associated with increases in the total colony area (fig. 7.5a). In PTH+Dx treated cultures these increases were dose dependent and in the 100nM Dx group, substantial (>100%). Early PTH treatment was associated with increases in the total colony area (fig. 7.5b). In PTH+Dx treated cultures these increases were dose dependent and in the 100nM Dx group, substantial (>500%). The total areas in the early PTH±Dx treated cultures was greater than those of the continuously treated.

7.3.6: AP+ Colonies.

Irrespective of the presence or absence of Dx, treatment with PTH whether continuous or early resulted in a significant increase ($p < 0.05$) in the number of AP+ colonies that formed (figs. 7.6a-d; 7.7a,b). Independently of the presence or absence of Dx, the total number of colonies formed in the cultures treated early with PTH was significantly greater than that of cultures treated with the hormone continuously.

7.3.7: AP+ Colony Mean Area.

AP+ colony area was not affected by any treatment (figs. 7.8a,b).

7.3.8: AP+ Colony Total Area.

Continuous PTH treatments were associated with increases in total colony areas (fig. 7.9a). In PTH+Dx treated cultures these increases were dose dependent and at 100nM, substantial (>200%). Early PTH treatment was associated with increases in the total colony area (fig. 7.9b). In PTH+Dx treated cultures a similar increase was seen in the 100nM Dx group while an increase in the 10nM Dx was considerable.

7.3.9: Percentage of Colonies Expressing AP.

Early but not continuous, treatment with PTH was associated with a significant increase ($p < 0.05$) in the proportions of AP+ colonies that formed (fig. 7.10a,b). This was the case whether with or without Dx.

7.3.10: Flow Cytometry.

Continuous treatment with PTH was associated with increased proportions of cells in the STRO-1-/AP+ population (figs. 7.11b-d; 7.15a). PTH+Dx treatment increased the proportions of cells in the STRO-1-/AP+ population and reduced the proportions of STRO-1-/AP- cells (figs. 7.12b-d; 7.15b).

Early PTH treatment with 100nM increased the proportions of cells in the STRO-1-/AP+ sub-population and reduced the proportions in the STRO-1-/AP- group (figs. 7.13b,d; 7.16a). 100nM PTH+Dx considerably increased the percentage of cells in the STRO-1-/AP+ population and reduced the proportion of cells in the STRO-1-/AP- group (figs. 7.14b,d; 7.16b).

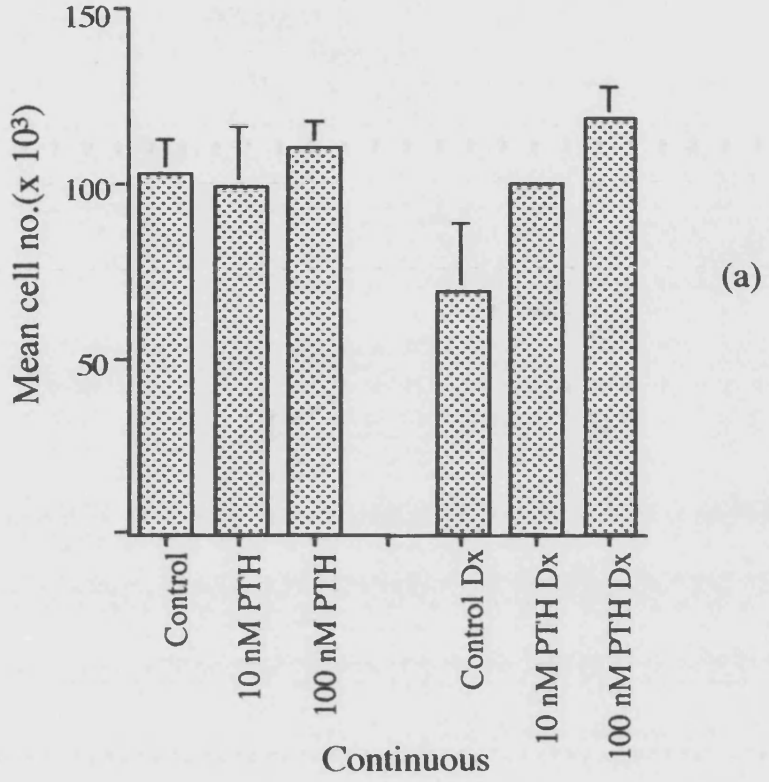
7.3.11: Expression of OC, BSP, CBFA1.

The expression of mRNA transcripts for OC, BSP and CBFA1 was detected under all conditions of culture. In the absence of Dx, treatment with PTH whether early or continuous did not effect their steady state level of expression (not shown). In the presence of Dx however, (fig. 7.17) steady state levels of expression were greater, and treatment with PTH was associated with an increase in the steady state level of BSP transcript, which was considerably greater in cultures exposed to the hormone for the first 48 hrs only. Treatment with PTH whether early or late was associated with a marked up-regulation in the steady state level of transcripts for CBFA1.

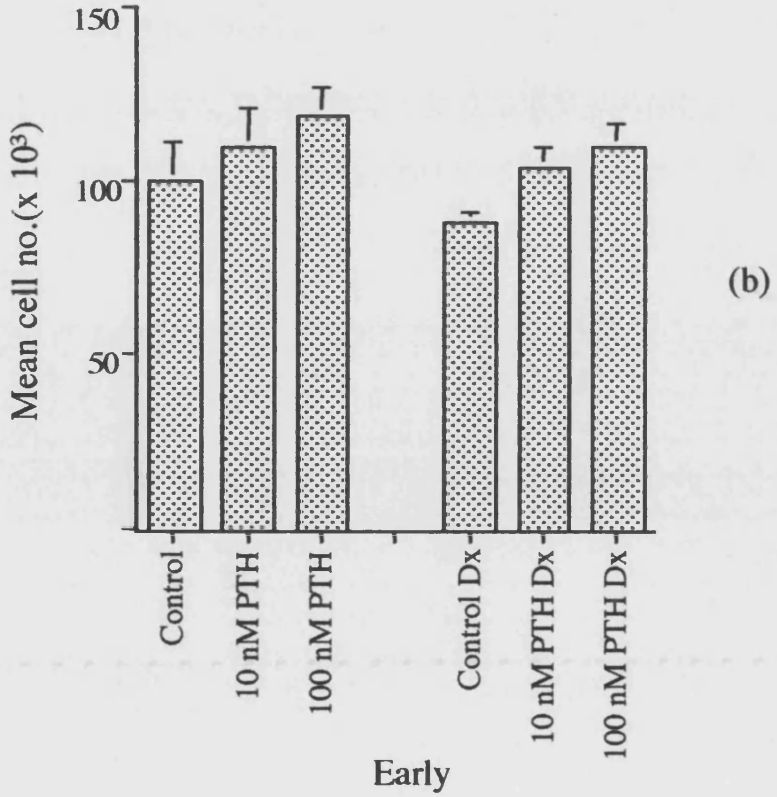
7.3.12: PTHr Expression and Intracellular cAMP Levels.

Treatment with Dx consistently increased the percentage of cells expressing the PTHr and increased cAMP response to PTH treatment (Table 7.1).

Figure 7.1 : Effect of PTH±Dx Treatment on Cell Number, (a) Continuous and (b) Early.



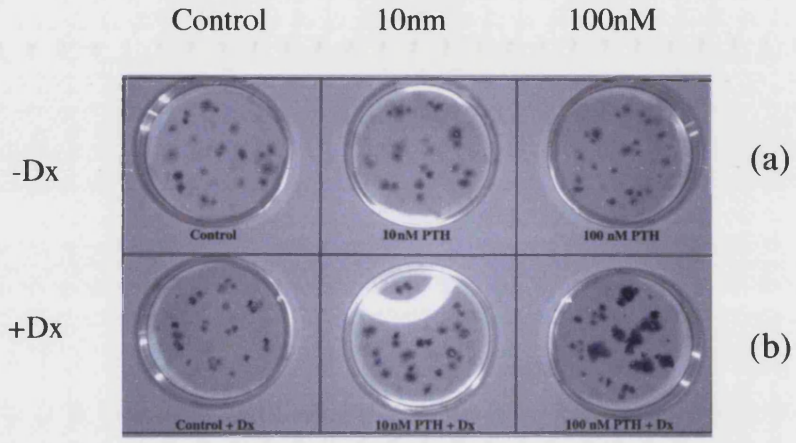
(a)



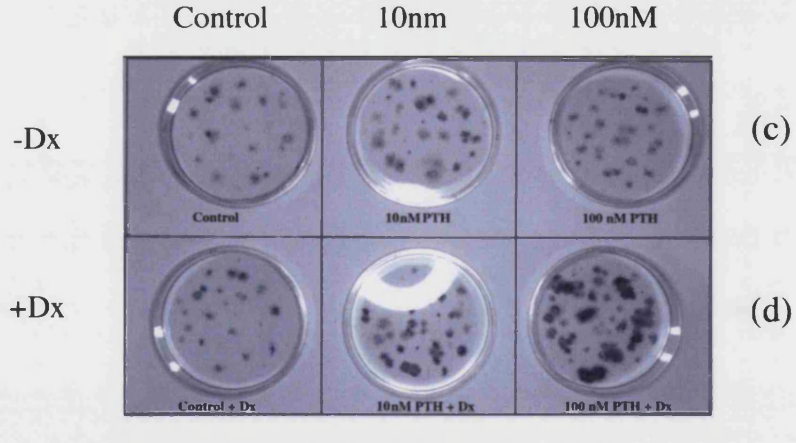
(b)

Figure 7.2: Photographs showing colony coverage at 21 days in response to continuous treatment with (a) PTH; (b) PTH Dx. and early treatment with (c) PTH; (d) PTH Dx.

Figure 7.2: Effect of PTH±Dx Treatment on Colony Number, (a,b) Continuous and (c,d) Early..



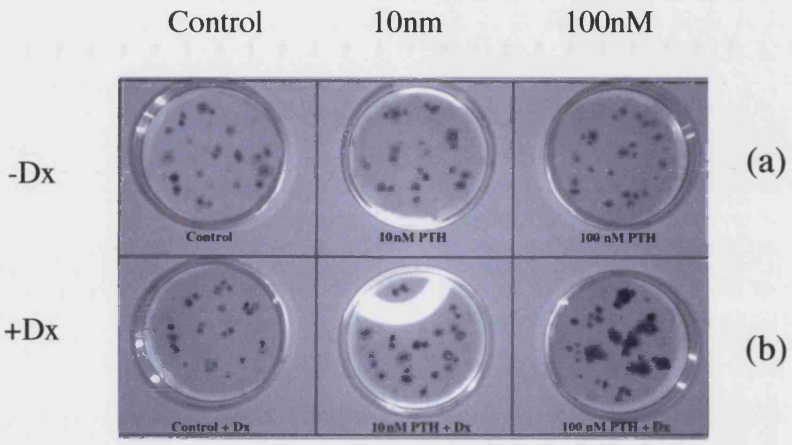
Continuous



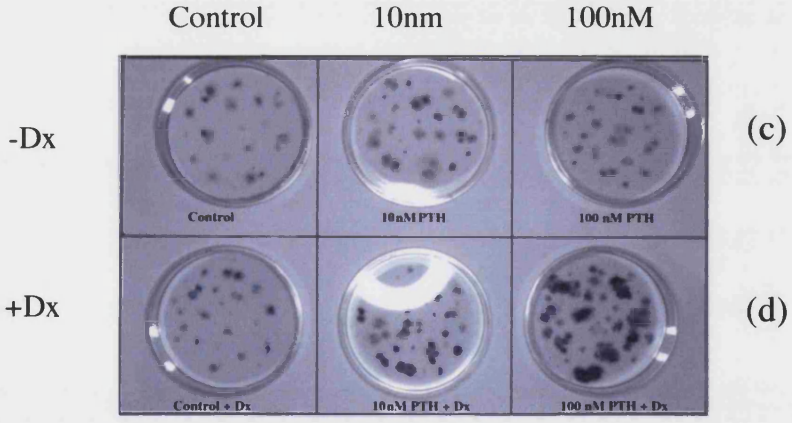
Early

Figure 7.2: Photographs showing colony coverage at 21 days
in response to continuous treatment with (a) PTH; (b) PTH Dx.
and early treatment with (c) PTH; (d) PTH Dx.

Figure 7.2: Effect of PTH±Dx Treatment on Colony Number, (a,b) Continuous and (c,d) Early..

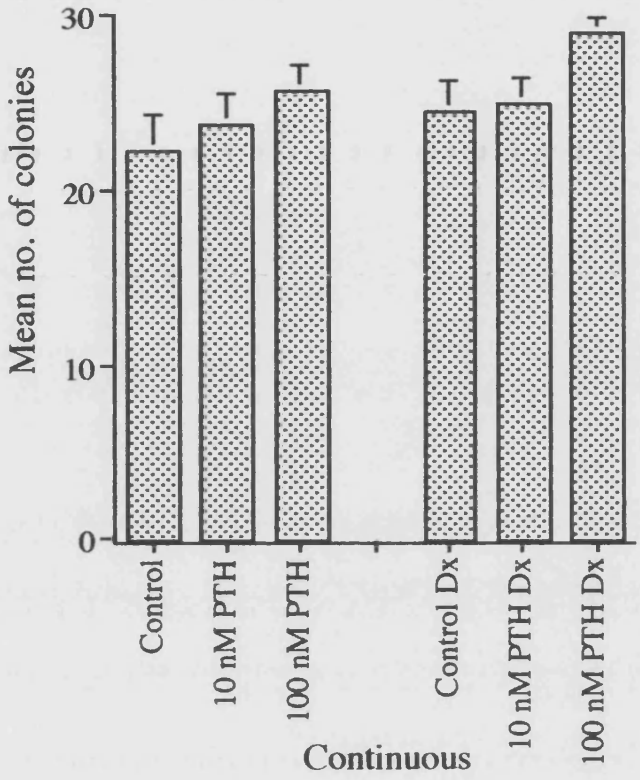


Continuous

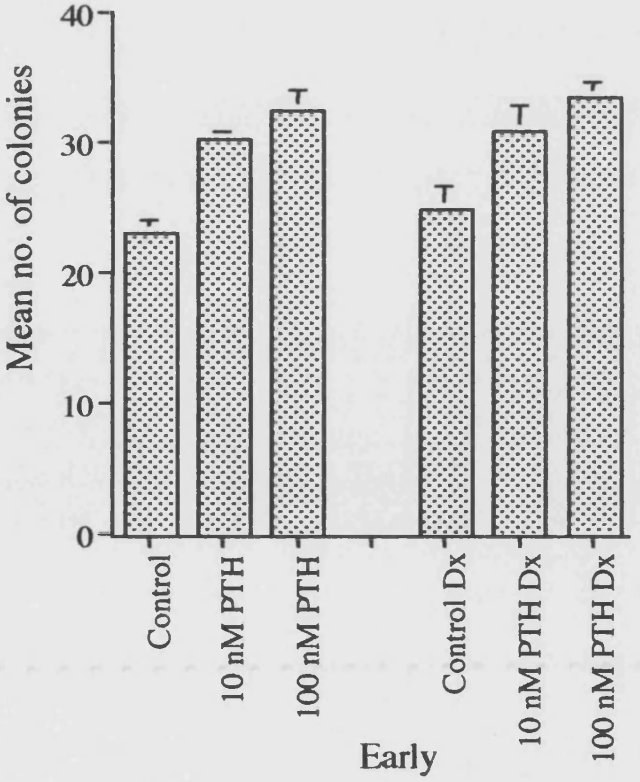


Early

Figure 7.3: Effect of PTH±Dx Treatment on Colony Number, (a) Continuous and (b) Early.



(a)



(b)

Figure 7.4: Effect of PTH±Dx Treatment on Mean Colony Area, (a) Continuous and (b) Early.

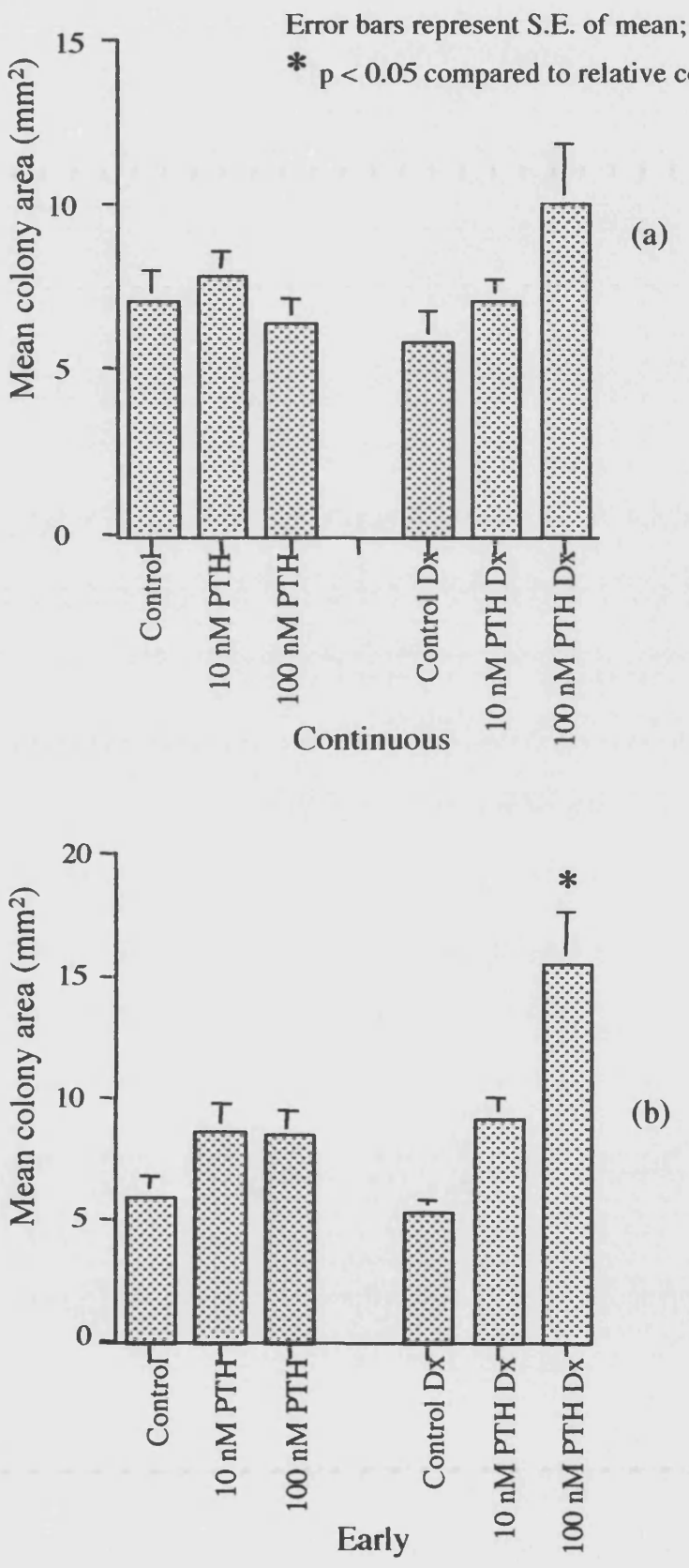
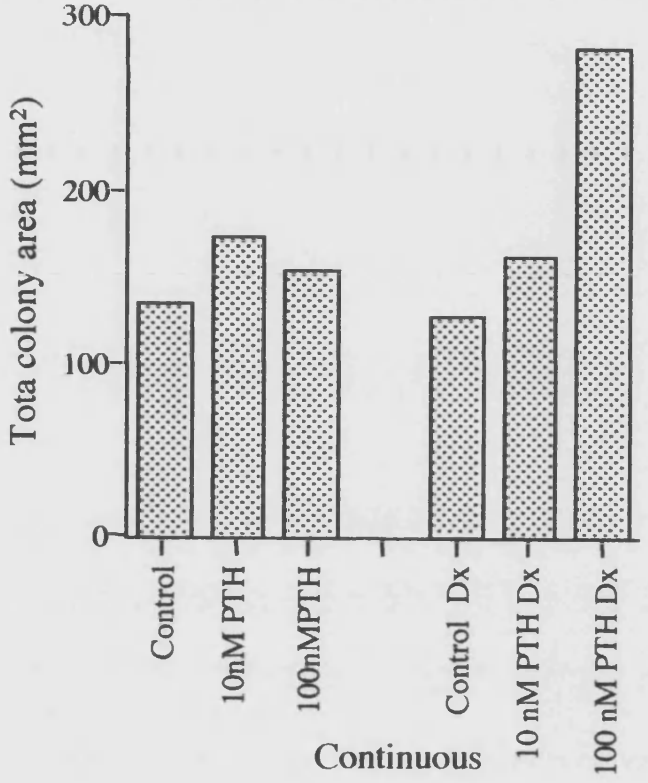
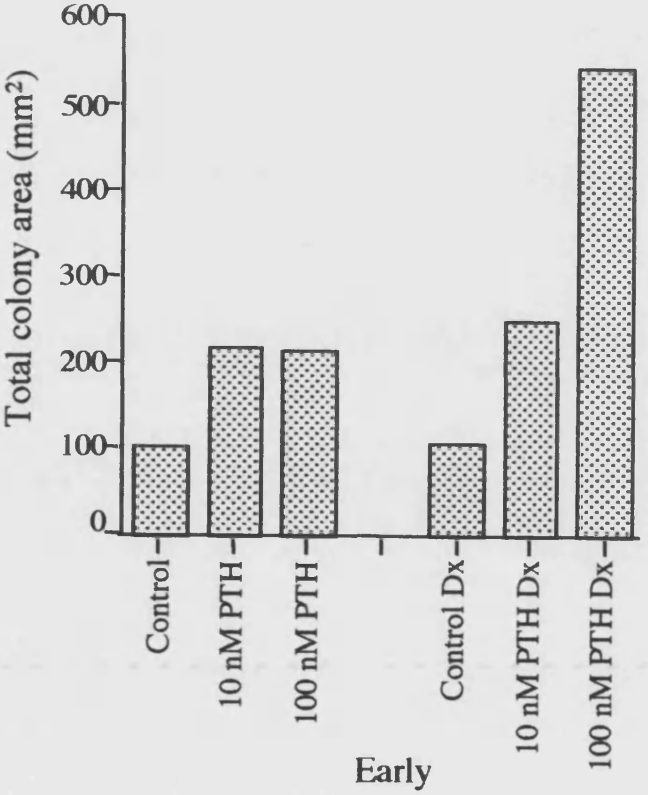


Figure 7.5: Effect of PTH±Dx Treatment on Total Colony Area, (a) Continuous and (b) Early.



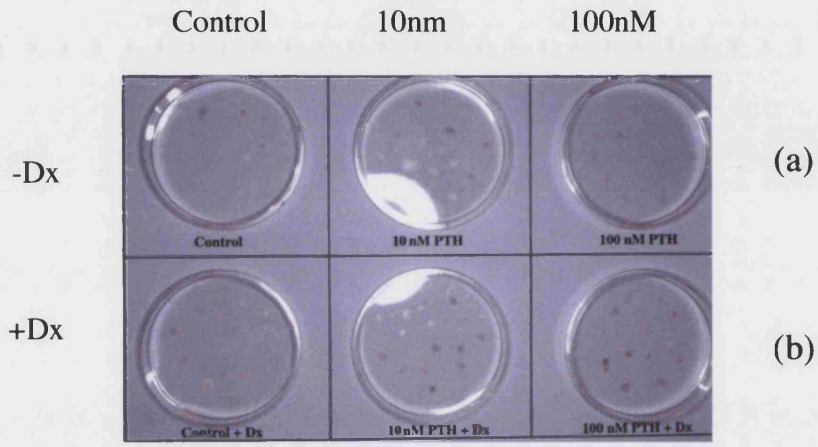
(a)



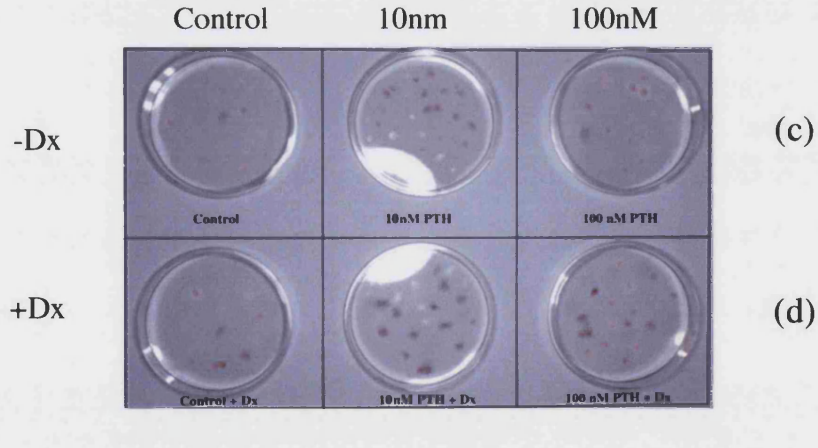
(b)

Figure 7.6: Photographs showing AP+ colony coverage at 21 days in response to continuous treatment with (a) PTH; (b) PTH Dx. and early treatment with (c) PTH; (d) PTH Dx.

Figure 7.6: Effect of PTH±Dx Treatment on AP+ Colony Number, (a, b) Continuous and (c, d) Early.



Continuous



Early

Figure 7.7: Effect of PTH±Dx Treatment on AP+ Colony Number, (a) Continuous and (b) Early.

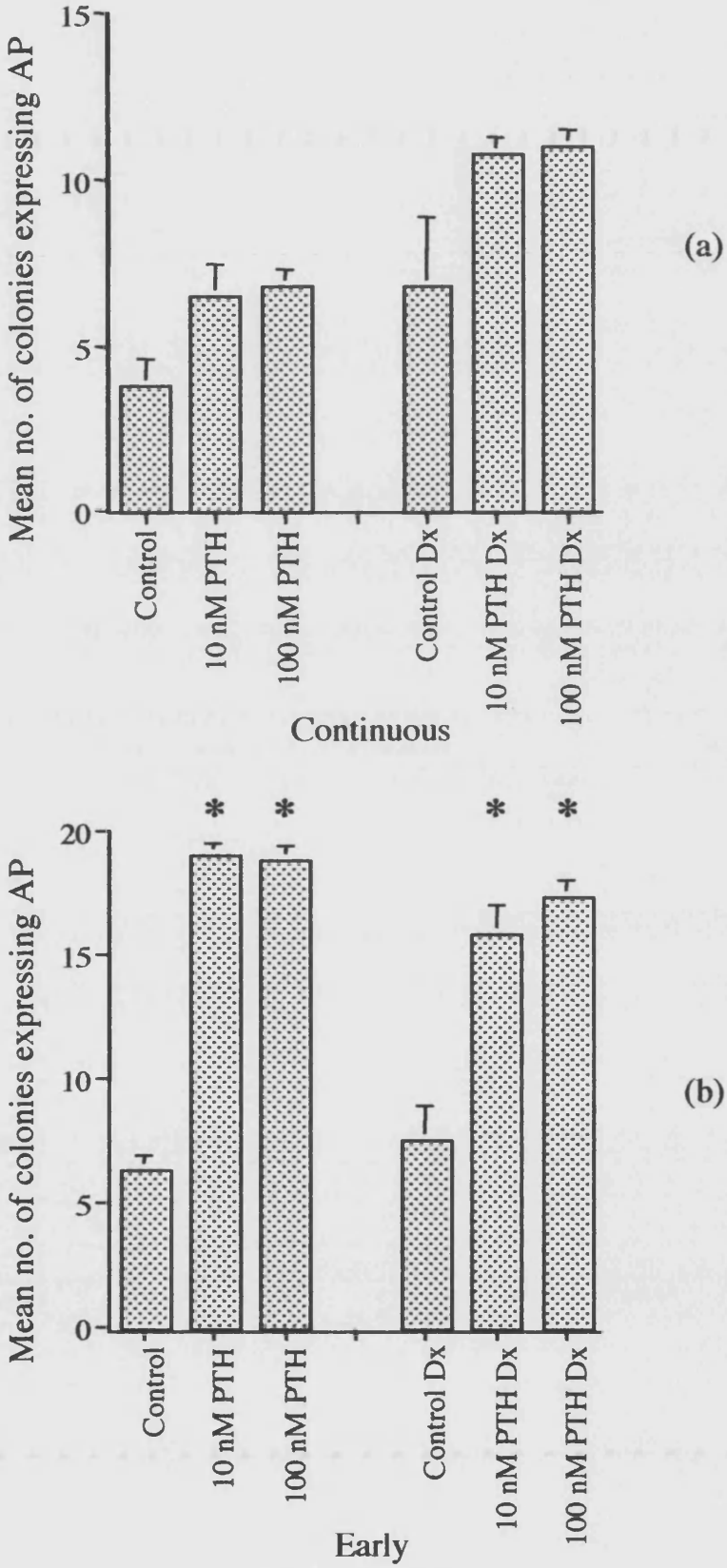
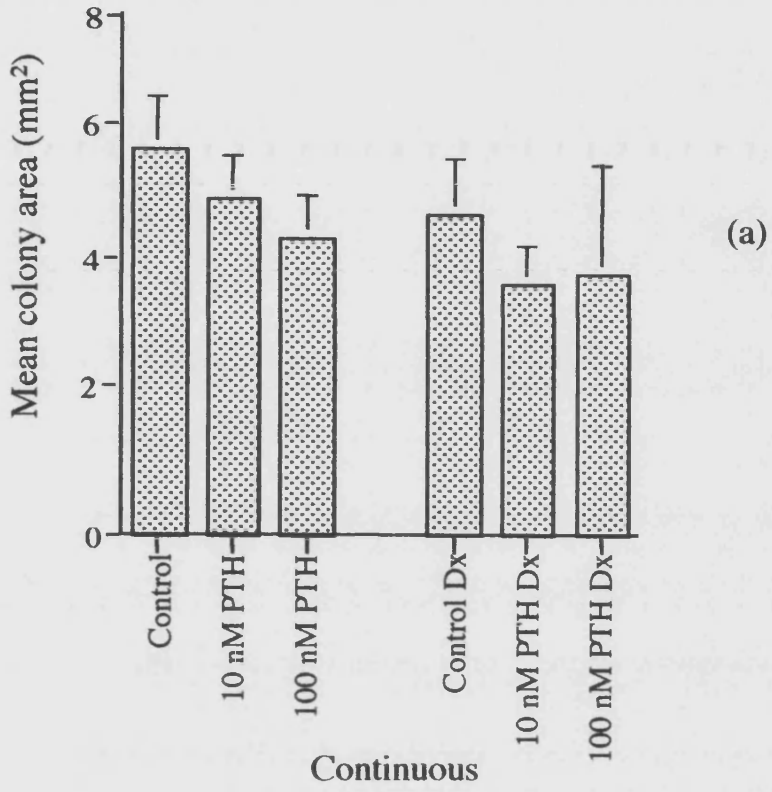


Figure 7.8: Effect of PTH±Dx Treatment on AP+ Mean Colony Area.
(a) Continuous and (b) Early.



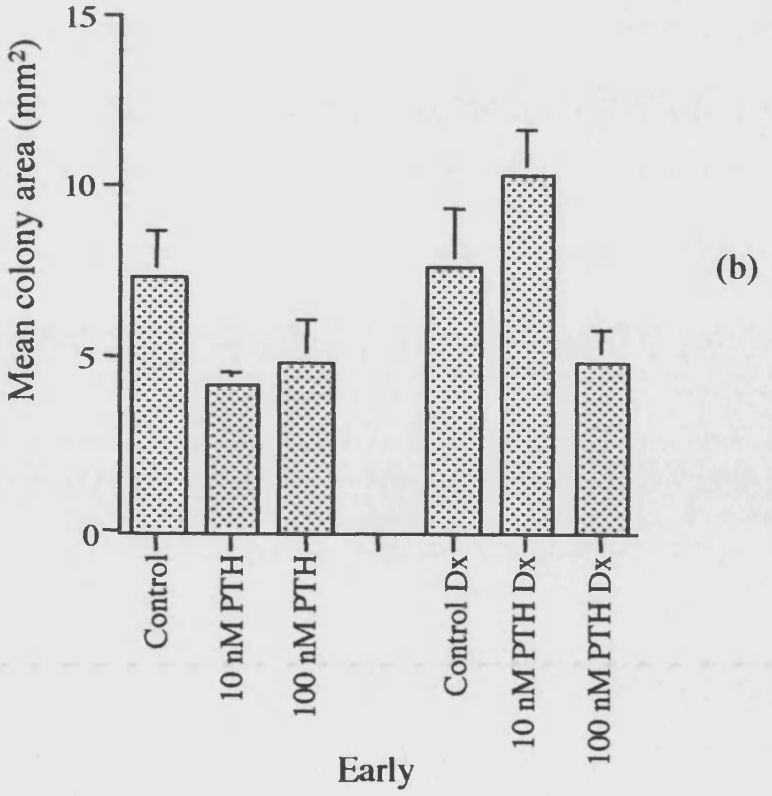
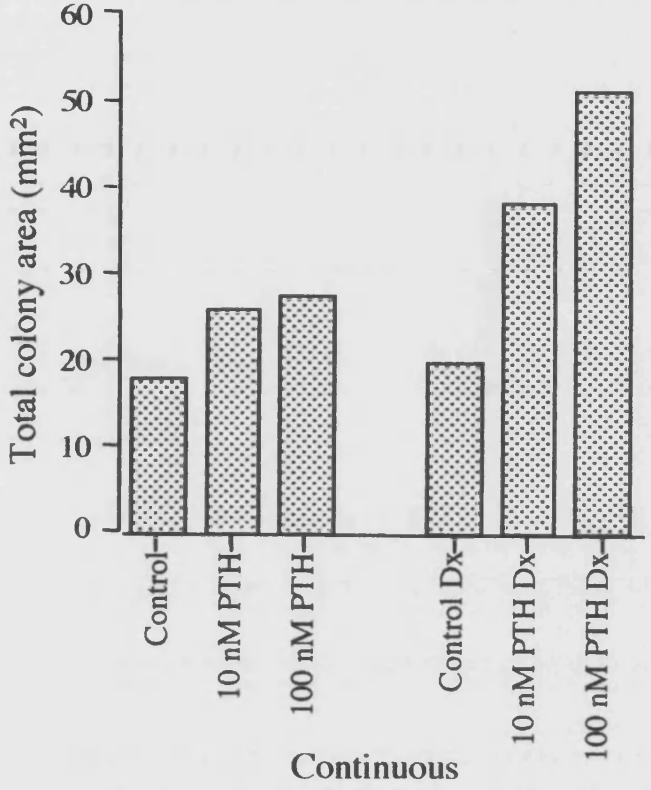
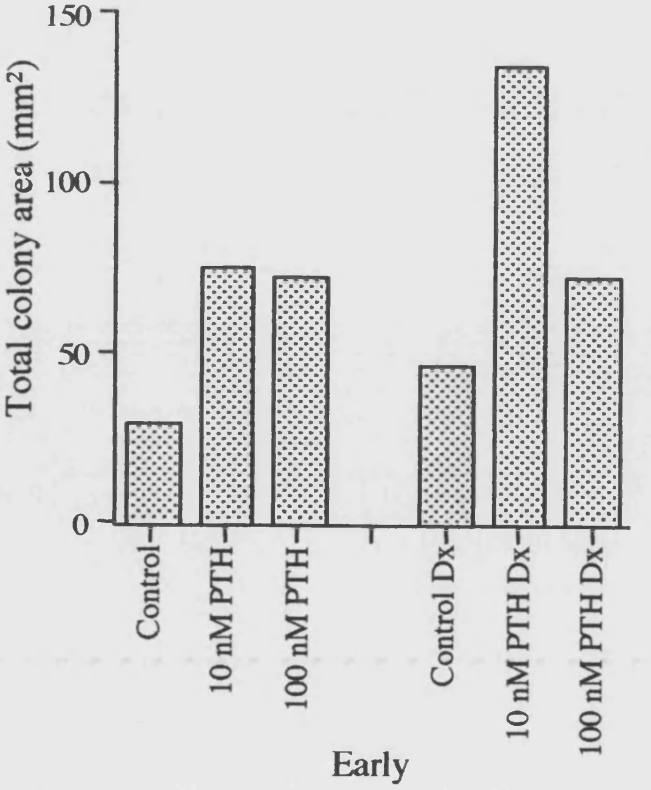


Figure 7.9: Effect of PTH±Dx Treatment on AP+ Total Colony Area, (a) Continuous and (b) Early.



(a)



(b)

Figure: 7.10: Effect of PTH±Dx Treatment on Percentage of Colonies Expressing AP, (a) Continuous and (b) Early.

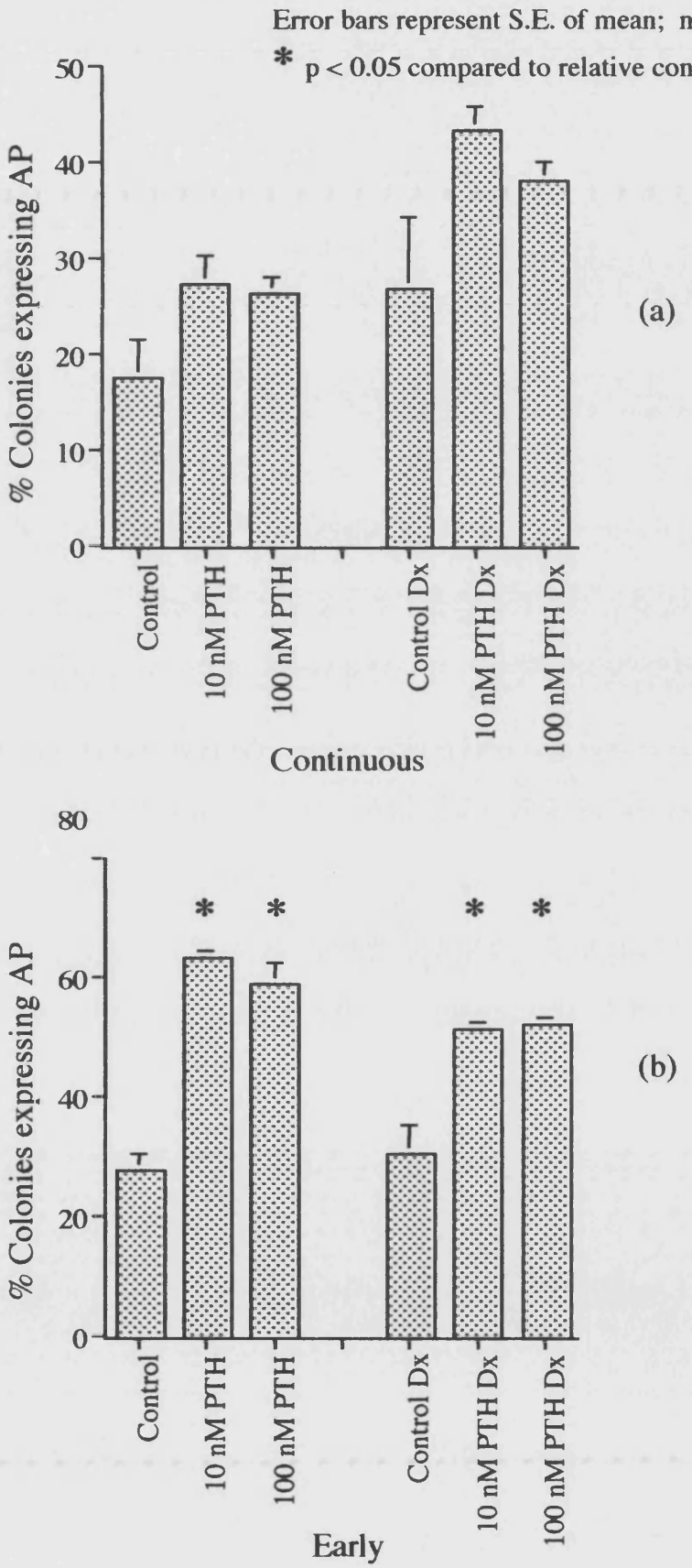
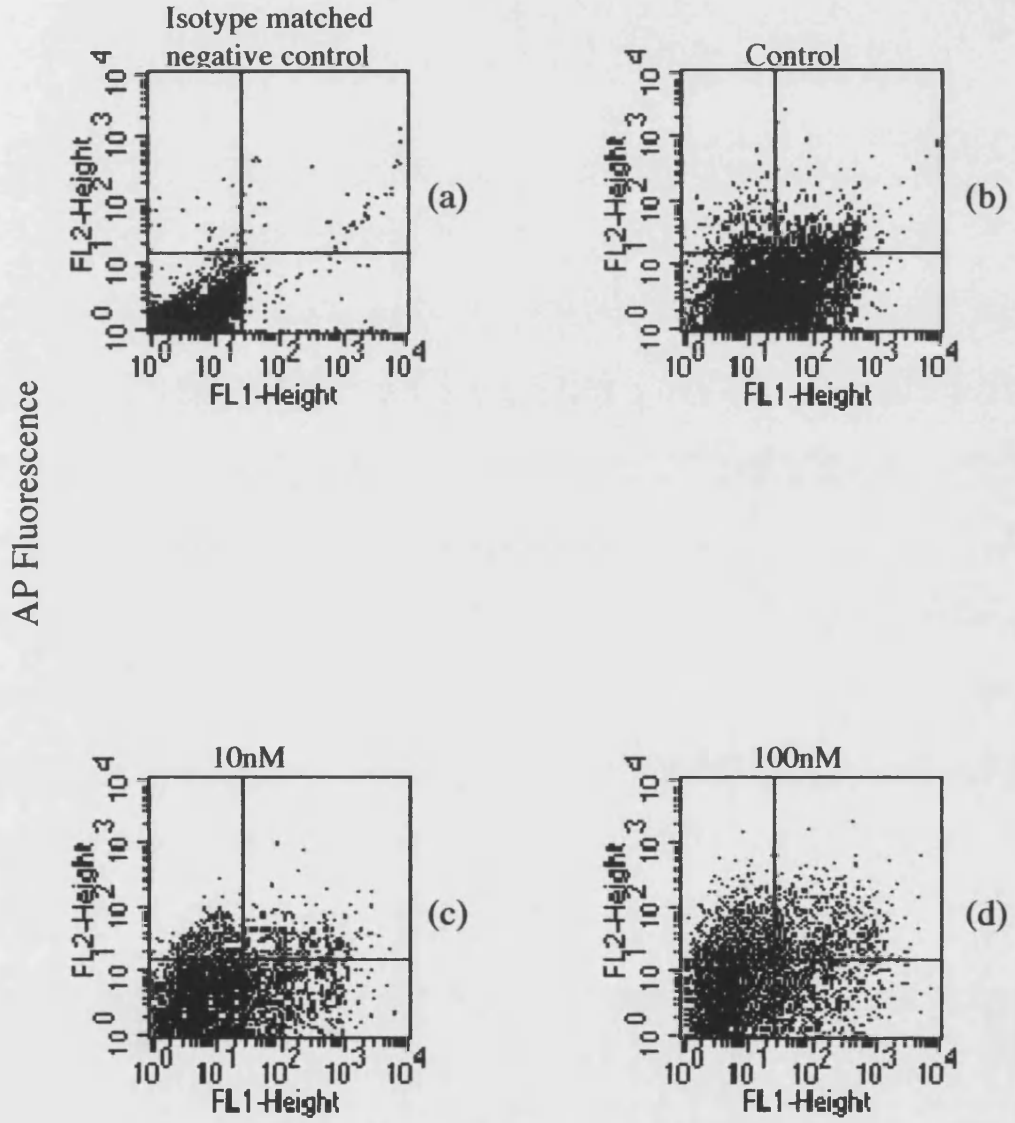
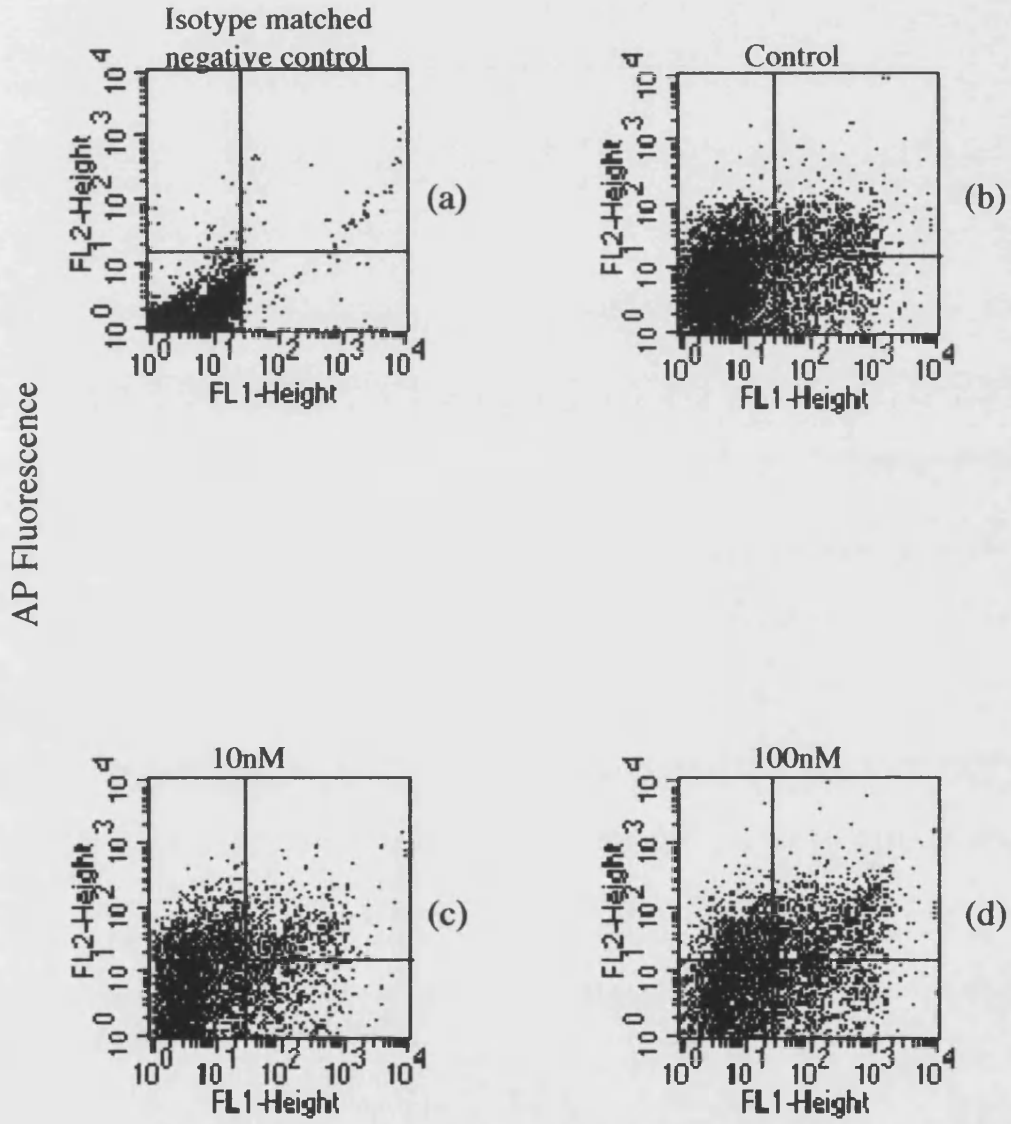


Figure 7.11 (a-d): Effect of Continuous PTH Treatment on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.



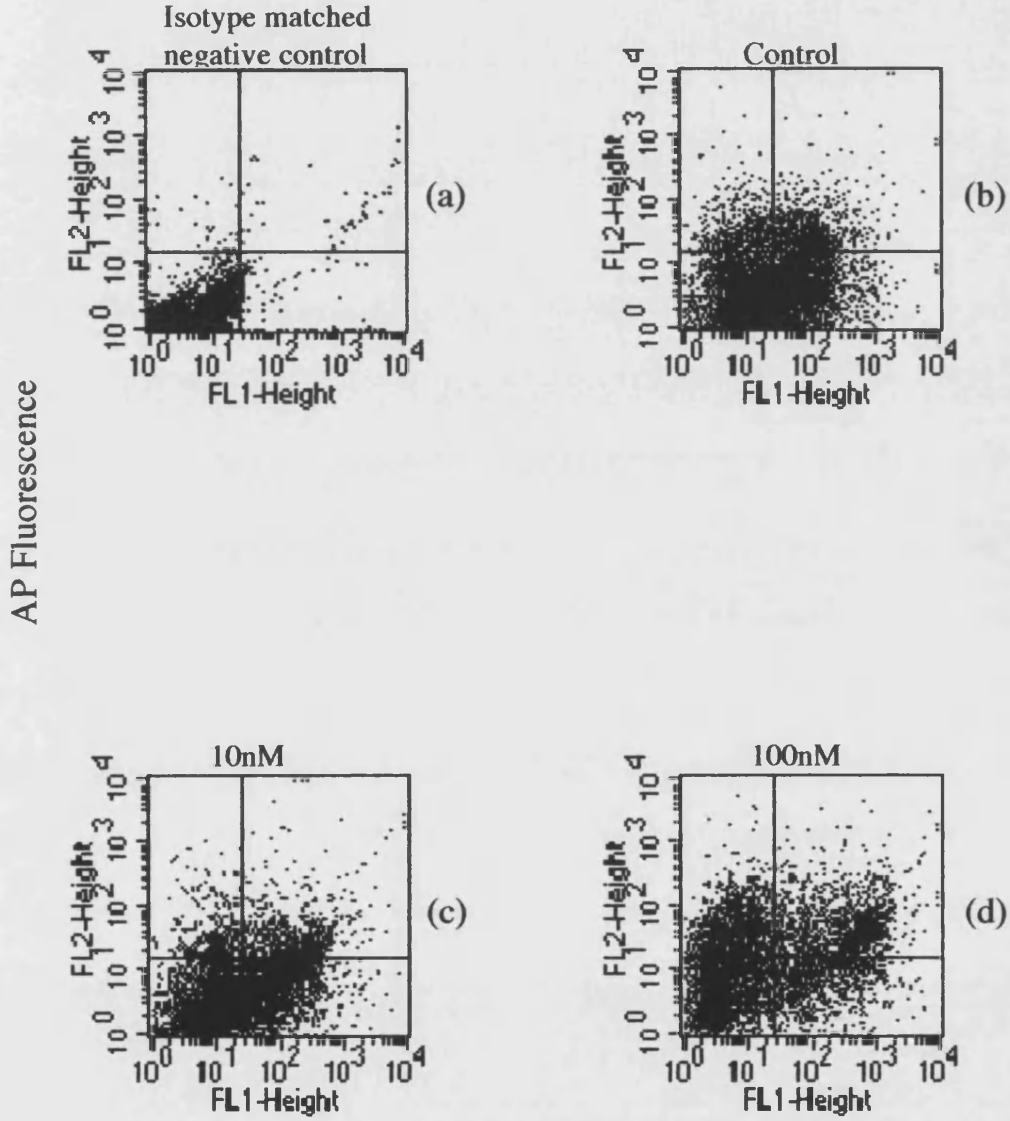
Cells that had been dual-labelled for expression of STRO-1 and AP antigens were visualised as dot plots with green fluorescence (FITC) on the *x* -axis and red fluorescence(R-PE) on the *y*--axis. Quadrants were set using the negative control sample such that >95% of cells appeared within the lower left quadrant (a).

Figure 7.12 (a-d): Effect of Continuous PTH Dx Treatment on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.



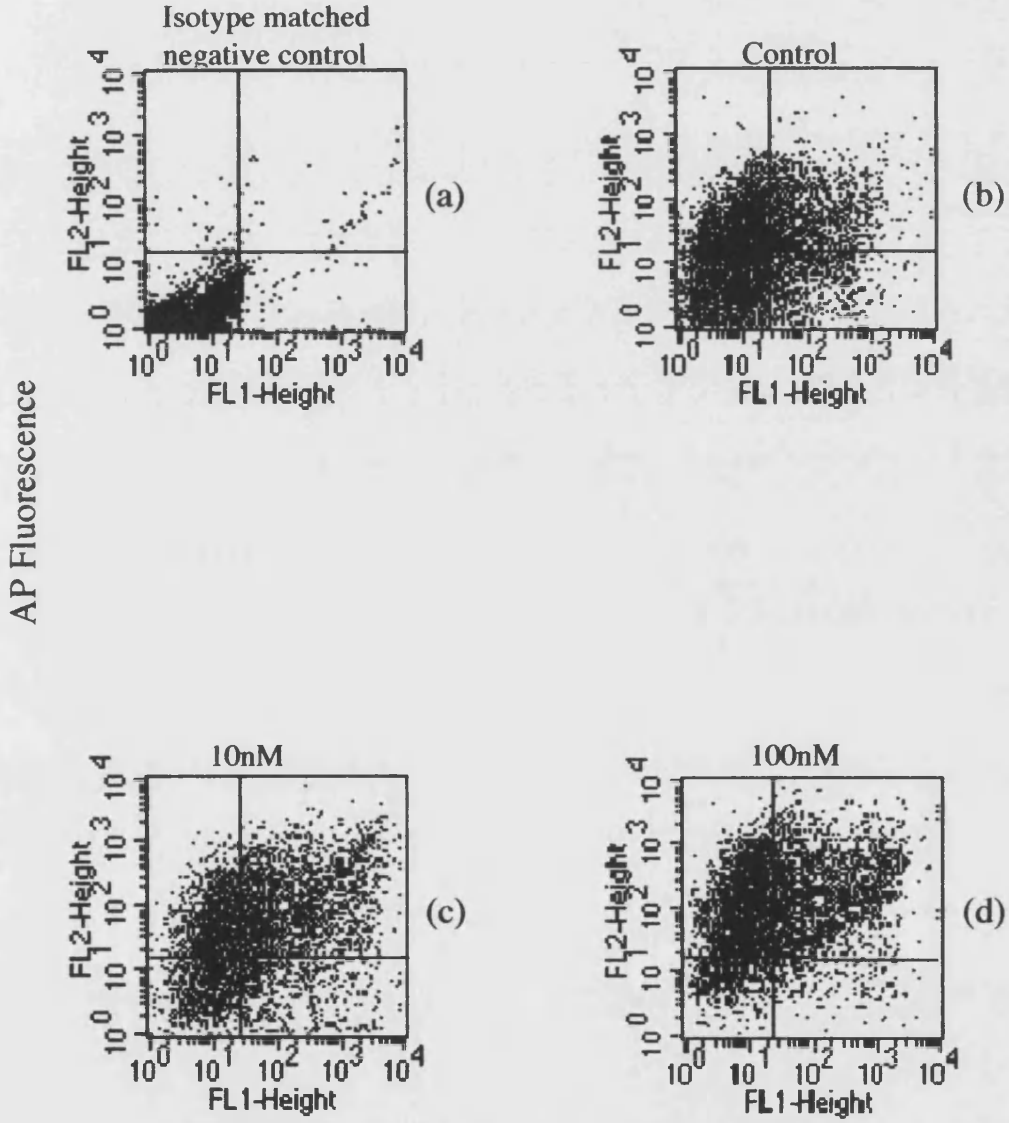
Cells that had been dual-labelled for expression of STRO-1 and AP antigens were visualised as dot plots with green fluorescence (FITC) on the *x* -axis and red fluorescence(R-PE) on the *y*--axis. Quadrants were set using the negative control sample such that >95% of cells appeared within the lower left quadrant (a).

Figure 7.13 (a-d): Effect of Early PTH Treatment on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.



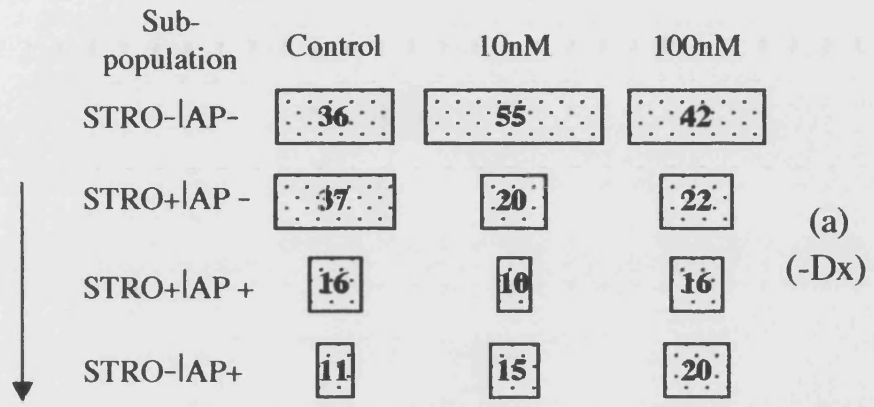
Cells that had been dual-labelled for expression of STRO-1 and AP antigens were visualised as dot plots with green fluorescence (FITC) on the *x* -axis and red fluorescence(R-PE) on the *y*--axis. Quadrants were set using the negative control sample such that >95% of cells appeared within the lower left quadrant (a).

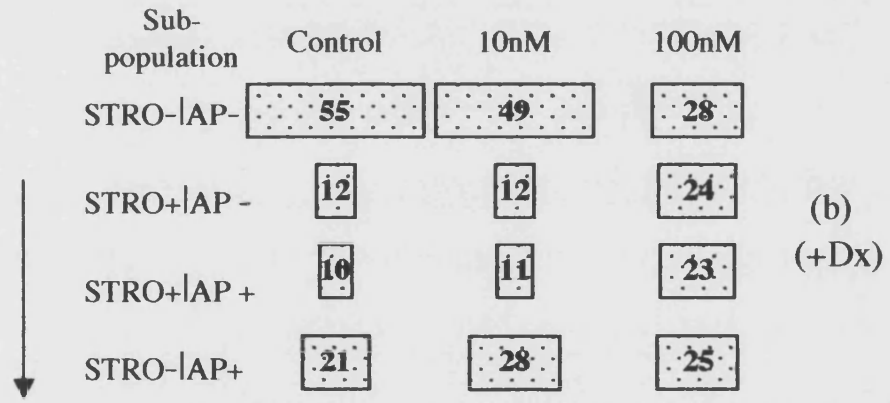
Figure 7.14 (a-d): Effect of Early PTH Dx Treatment on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.



Cells that had been dual-labelled for expression of STRO-1 and AP antigens were visualised as dot plots with green fluorescence (FITC) on the *x* -axis and red fluorescence(R-PE) on the *y*--axis. Quadrants were set using the negative control sample such that >95% of cells appeared within the lower left quadrant (a).

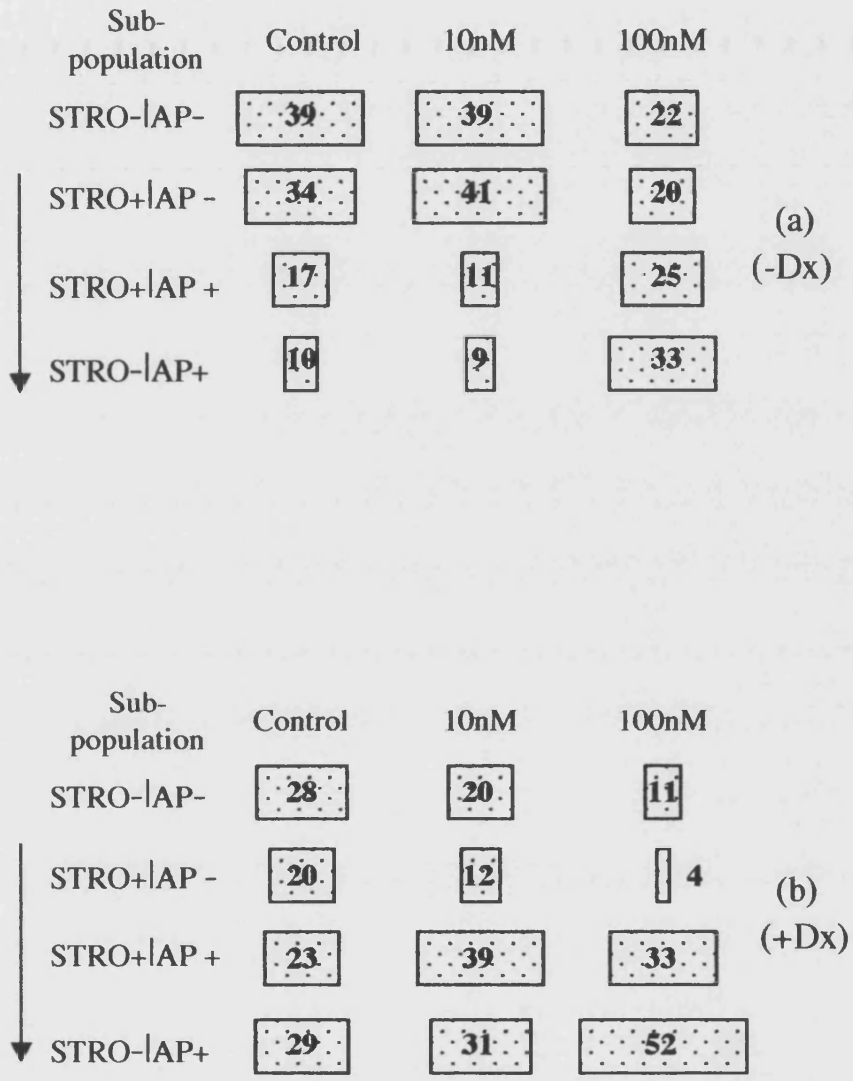
Figure 7.15: Effect of the Continuous Treatment with (a) PTH and (b) PTH Dx on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.





Relative proportions of sub-populations of cells labelled with the STRO-1 and AP antibodies. Block areas represent relative sub-population sizes, numbers associated with blocks are percentage of cells (to nearest percent). Arrows represent the direction of increasing osteogenic differentiation.

Figure 7.16: Effect of the Early Treatment with (a) PTH and (b) PTH Dx on the Co-Expression of STRO-1 and AP Antigens as Assessed by Flow Cytometry.



Relative proportions of sub-populations of cells labelled with the STRO-1 and AP antibodies. Block areas represent relative sub-population sizes, numbers associated with blocks are percentage of cells (to nearest percent). Arrows represent the direction of increasing osteogenic differentiation.

Figure 7.17: Expression of osteoblast related transcripts of BSP, OC, CBFA1. Figure shows Southern Blots of the RTPCR products probed with specific oligos recognising sequences internal to those used for the PCR.

Figure 7.17:Effect of Treatment with PTH on theExpression of Osteoblast-Related Genes in Dx Treated Cultures of BMSC.

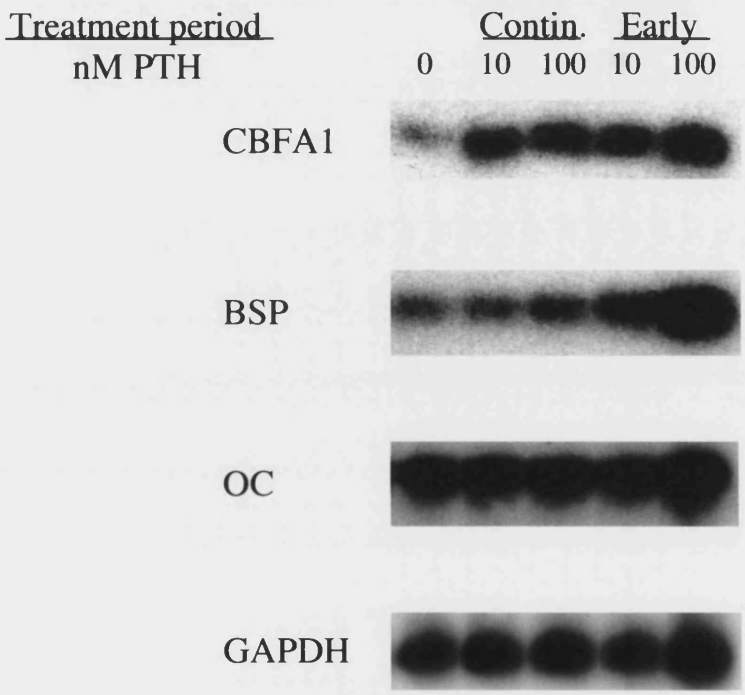


Table 7.1: Effect of PTH on the Intracellular Accumulation of cAMP.

			% Cells PTHr +ve		MFI		cAMP response to PTH and Dx treatment,- % of non-stimulated control		
Donor	Sex	Age	-Dx	+Dx	-Dx	+Dx	+Dx	+PTH	+PTH+DX
1	F	73	32.3	49.6	16.4	16.3	81± 9.4	114± 2.8	366±100.8
2	M	70	43.6	58.1	12.9	12.5	126±18.8	138±16.1	621± 61.8
3	M	52	83.4	85.5	35.2	47.8	175±12.1	106±15	738± 68.7

7.4: Discussion.

Both continuous and early treatment with PTH caused changes in BMSC morphology similar to that seen in the previous experiments. Considering these observations along with the effects witnessed in the late treated cultures of the previous experiment suggests that PTH must be in the culture system early in order to affect cell morphology. The results of this experiment add weight to the suggestion that PTH targets less differentiated cells[296] while concomitantly inhibiting the differentiation and proliferation of more cells at later stages for differentiation[144,294,300].

The effect of PTH treatments on cell numbers in the continuous treatments was similar to that seen in the previous experiment and where PTH was supplemented with Dx, the significant increase seen in the 100nM Dx was in accordance with the previous findings. Consistent with the previous experiments, PTH treatment increased the number of colonies and this increase was also apparent when Dx was added to the system. When PTH±Dx was added early, the increases in colony numbers was greater than that seen in the continuously treated. This increase might be explained by the model discussed previously. (i) The presence of PTH at initiation of cultures recruits un-committed/non-adherent cells and promotes them to adherent/committed types. (ii) The presence of PTH in the post adherence stage acts to inhibit the proliferation and differentiation of these recruits and other adherent cells. An analysis of the mean colony areas, an indicator of colony proliferation[180], supports this thesis, in cultures where PTH was applied (and removed) early the mean colony area was greater than that of the comparable continuous treatments.

Consistent with the previous experiment, treatment with PTH±DX caused a significant increase in the number of AP+ colonies. When treatment was applied early this increase was significantly greater. Expressing this increase as a percentage of the total colonies reveals significantly higher values in the early treatments. This increase in AP+ seen in the early treatments might be explained if PTH preferentially recruited a sub-population of non-adherent/uncommitted cells which had a greater osteogenic potential. It might also be explained by a PTH induced increase in osteogenic differentiation although previous studies in this area suggest that this is not the case[144,294,300]. Early treatment with PTH±Dx at 100nM produced meaningful changes in the STRO-1/AP sub-populations which was suggestive of an increase in osteogenic differentiation. If it is accepted that PTH does not directly increase osteogenic differentiation *per se* then this effect might be explained by a PTH induced increase in the proportions of cells of the osteogenic phenotype.

Analysis of mRNA transcripts for osteocalcin (OC) bone sialoprotein (BSP), late stage markers of osteogenic differentiation[12,301,302] and CBFA1/OSF2, a transcription factor essential for osteogenic differentiation[210-212] reveals inter-treatment differences in mRNA levels for BSP and CBFA1 when Dx is present in the system. OC levels appeared to be unaffected by treatments, PTH has previously been shown to both inhibit OC production[303,304] and increase OC gene expression[305] so the finding of this study adds little to that debate. Treatment with PTH Dx both continuously and early, caused similar increases in levels of BSP mRNA, this increase is consistent with previous work on PTH effects[306] although it is not clear whether this upregulation is caused by an increase in osteogenic differentiation or, if PTH simply causes an increase in levels of this protein.

mRNA for the transcription factor CBFA1 was upregulated by continuous treatment at 100nM Dx and by early treatment of PTH Dx. In osteogenesis, CBFA1 is a crucial transcription factor that induces osteoblast related genes[209] and has the ability to up-regulate these genes in pre-osteoblastic or non-osteoblastic cells[210]. The upregulation of CBFA1 by PTH provides a mechanism by which PTH might recruit non-adherent/uncommitted cells to the osteogenic lineage and thereby increase the proportions of osteogenic cells in culture.

The interaction between PTH and Dx seen in this and other studies[307-310] can be partly explained by Table 7.1. This table shows the effects of Dx on PTHr expression and the associated cAMP response in BMSC from three donors. The addition of Dx consistently increased the proportion of cells expressing PTHr and concomitantly increased the cAMP response when Dx was applied with PTH. This finding is in accordance with the suggestion that Dx increases the proportions of cells expressing PTHr by increasing the transcription rate of the PTHr gene[308]. The level of expression of PTHr per cell is indicated by the Mean Fluorescent Intensity (MFI) and in these samples this parameter does not seem to play a major part in the increase in cAMP response associated with the combination treatment. Dx might also potentiate hormone responsiveness through promotion of hormone-receptor-adenyl cyclase coupling[310] although the evidence derived from this experiment is not sufficient to allow comment on this possibility.

7.5: Summary.

The findings of the PTH study allows the definition of a simple model system:
Treatment with PTH at the initiation of culture promotes uncommitted/non-adherent cells to a more committed phenotype via PTH induced upregulation of CBFA1 gene

expression and thereby increases the pool of osteoprogenitors. The addition of Dx to the system enhances this effect by upregulating the proportions of cells expressing the PTHr1 receptor and potentiating the hormone-receptor-adenyl cyclase coupling[310]. Subsequent proliferation and differentiation of the adherent cells is inhibited by the presence of PTH, the level of inhibition increasing with differentiation[294,311,312]. The different actions of PTH which are seen at different levels of maturation could be due to the increasing expression of PTHr associated with increasing differentiation[279,313].

This model could be developed in a number of ways, optimisation of the duration of the early PTH treatment may reveal greater osteogenic effects. It is possible that an investigation of the correlation between PTHr expression and differentiation would provide clues as to the cause of the disparate effects seen with PTH treatments. Additionally, a knowledge of the relative contributions of the PKA/PKC signalling pathways to the differing effects could prove to be useful.

It is widely accepted that the intermittent application of PTH modulates anabolic effects which are dependent on the time and duration of treatments[314,315]. The simple model system defined in this study provides one possible explanation as to how intermittent treatments could give rise to an increased pool of osteoprogenitors thereby enhancing anabolic potential. Development of this system could provide a basis to explore the disparate effects evident between treatment regimes and may lead to an understanding of the underlying cellular and molecular events. Understanding these mechanisms may eventually contribute to the development of therapies for osteopenic conditions.

Chapter 8: Conclusions.

8.1: Conclusions.

Osteoporosis is a significant cause of morbidity and mortality in the United Kingdom. As a focus of research, a wide range of strategies have been implemented in an attempt to elucidate the mechanisms underlying this problem. Human studies and research using *in vivo* animal models have been complemented by *in vitro* molecular and cell biology techniques.

Stem cell recruitment and promotion are important modulators of bone formation and a defect in this system has been suggested as a contributory factor in the pathogenesis of this condition. The realisation that these cells can be modulated by growth factors and hormones at the earliest stages of development gives rise to hope of therapies based on this type of manipulation. To date, the understanding of osteoblast recruitment and differentiation is incomplete and this deficit in knowledge hinders the development of such therapies.

This study set out to examine the potential of modulating osteoblast differentiation using growth factors and hormones and to ascertain if the temporal application and combination of these agents could be used to further enhance osteoblast progression.

In this study samples were rejected if patients had evidence of conditions (e.g. carcinoma) or therapies (e.g. glucocorticoids) known to affect bone metabolism. Despite this exclusion criteria inter-sample variability was occasionally seen between patients who were age and sex matched. These differences might be attributed to factors outside our control e.g. inconsistencies in handling samples and differences in transit times, but it is equally likely that as yet unknown biological mechanisms are involved.

This study has shown;

- Choice of serum supplement has important effects on the proliferation and differentiation of bone marrow stromal cells.

- When applied early to BMSC, FGF is a mitogen and positively influenced osteogenic differentiation. Early treatment with FGF selectively increased the proportion of cells present in the multipotential precursor and committed osteoprogenitor populations.

- Treatment with PTH promoted osteogenic differentiation, possibly by promoting uncommitted stem cells to the osteogenic lineage. This enhancement was

increased when PTH was applied early and decreased when applied late. A positive osteogenic interaction between PTH and Dx was associated with a Dx related increase in the proportion of cells expressing PTHr1.

This study therefore, provides evidence that the manipulation of osteoblast differentiation at the stem cell level can be further enhanced by the temporal application of the growth factors, TGF-β and FGF-1 and the hormone, PTH. It is hoped that these findings may contribute to the understanding of bone stem cell recruitment and promotion, a process which is of fundamental importance to bone formation.

This type of study is limited in a number of ways. Samples of human marrow were obtained remotely from patients undergoing elective surgery and because of time constraints placed on surgical teams, it was not always possible to obtain full records of patient details. Often, only the minimum details of age, sex, disease status and current drug therapy were supplied. Ideally, the provision of a full medical history would allow the identification of previous conditions and this could add another dimension to the overall analysis.

8.2: Future Work.

In Vitro

- Further insights could be gained by expanding the temporal range of applications of these agents. A comparison of integrin expression and activation between treatment groups may provide clues to underlying mechanisms governing the promotion of non-adherent to adherent cells.

In Vivo

- These treatments, when applied in a suitable animal model, would allow the investigation of histomorphometric parameters.

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Appendix.

Table 1: Age and Gender Details of Patients used in this Study.

Chapter	Sex	Age
3: Testing of Serum Supplements	Male	59
3: Testing of Serum Supplements	Female	68
3: Testing of Serum Supplements	Female	68
4: Effects of TGFβ-1 and FGF-2 Treatment	Female	68
5: Effect of PTH Treatment	Female	68
6: Effect of Delayed PTH Treatment	Unknown	31
7: Effect of Early PTH Treatment	Male	66
7: Effect of Early PTH Treatment	Male	52
7: Effect of Early PTH Treatment	Male	73
7: Effect of Early PTH Treatment	Female	65